Physiological and genetic control of anthocyanin pigmentation in different species

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Chapter 4

Transcriptional analysis in high-anthocyanin tomatoes reveals synergistical 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 overrepresented 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.

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). Besides providing attractive colours 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 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, *SlAN2* (Mes et al., 2008; Boches et al., 2009) and *Anthocyanin 1* (*SlANT1*) (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, thus revealing sets of differentially regulated genes and synergistical effects of *Aft* and *atv*. Our results demonstrate that the regulation of transcripts involved in the phenylpropanoid metabolism is tightly linked to the anthocyanic phenotypes of tomato fruits, and is also accompanied by changes in other structural and metabolic traits.

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

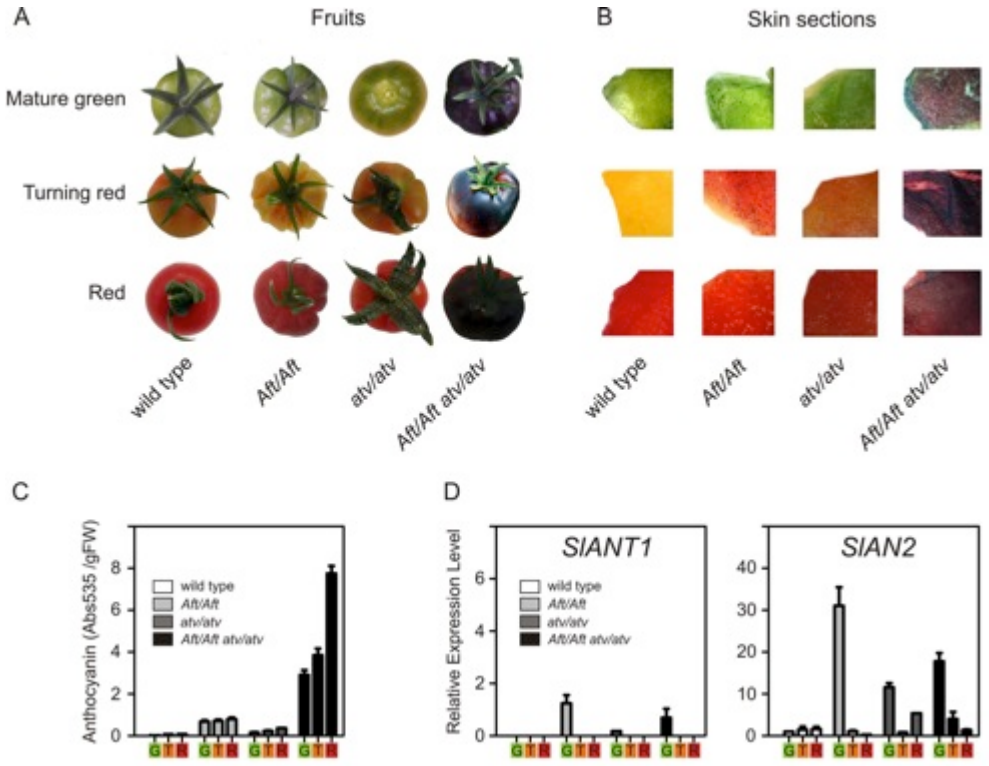

Figure 1. Anthocyanin production in different tomato genotypes.
(A) Mature green, turning red and red tomato fruits.
(B) Fruit skin sections taken from Ailsa Craig (AC), *Aft/Aft*, *atv/atv*, and *Aft/Aft atv/atv* plants.
(C) Anthocyanin levels in tomato peel from AC, *Aft/Aft*, *atv/atv*, and *Aft/Aft atv/atv* fruits (data are means of three replicates ±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) *SlAN2* 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 *SlAN2* 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 ±SD.

AC and *atv/atv* fruits (Figure 1A, B), whereas *Aft/Aft* fruits showed purple spots in their peel since the G stage (Figure 1A, B).

As expected, a very strong anthocyanin pigmentation was displayed by *Aft/Aft atv/atv* fruits (Figure 1A, B). The presence of anthocyanins was evident since the G stage and limited to the fruit peel (Figure 1A, B), while they were not produced in the flesh

Figure 2. Cross section of fruits harvested from the *Aft/Aft atv/atv* double mutant. Anthocyanins are concentrated in the peel, while they are not produced in the flesh.

(Figure 2). Anthocyanin quantification confirmed the constant accumulation of pigments during fruit ripening in *Aft/Aft* and, especially, in *Aft/Aft atv/atv* (Figure 1C). Very small amounts of anthocyanins, detectable only by spectrophotometric analysis, were also measured in *atv/atv* fruits (Figure 1C). On the other hand, no significant anthocyanin production was measured in AC tomatoes (Figure 1C), or in the flesh of the fruit from all the genotypes analyzed (data not shown).

The strong anthocyanin accumulation in *Aft/Aft atv/atv* fruits (Figure 1A, 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 homogenously throughout the skin, thus conferring a purple colour to the epidermis of the fruit (Figure 1A, B).

***SlANT1* and *SlAN2* 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 clues regarding the transcriptional regulation of anthocyanin synthesis in tomato, the mRNA levels of *SlANT1* and *SlAN2* 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 *SlANT1* and for *SlAN2* was observed in *Aft/Aft*, *atv/atv* and *Aft/Aft atv/atv* fruits at the G stage (Figure 1D). On the other hand, *SlAN2* and *SlANT1* expression was negligible in the peel of AC (Figure 1D) and in the flesh of all the genotypes (data not shown), where anthocyanins were not produced. The relative expression levels of *SlAN2* and *SlANT1* 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 *SlANT1* and/or *SlAN2* genes might be candidates for the *Aft* mutation (Sapir et al., 2008; Boches et al., 2009). Both *SlANT1* and *SlAN2* were expressed in all the genotypes that produce anthocyanins in the fruit skin. However, their expression declined during the T and R phases (Figure 1D). Anthocyanin content, instead, increased throughout ripening in *Aft/Aft atv/atv* fruits (Figure 1C). It is likely that, after the G phase, *SlANT1* and *SlAN2* are replaced, recruited

and/or inhibited by other transcription factors, thus becoming less crucial for further anthocyanin production and accumulation.

As a whole, our data suggest that *SlANT1* and *SlAN2* might act as early triggers of anthocyanin production, particularly in green fruits. At later stages of ripening, other factors are likely necessary for promoting a strong and continuous anthocyanin accumulation in tomato skin.

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 (Figure 3A), LBGs are more specific to a restricted number of groups, including anthocyanins (Figure 4A). The analysis of the biosynthetic pathway in tomato fruits was therefore subdivided into these two parts.

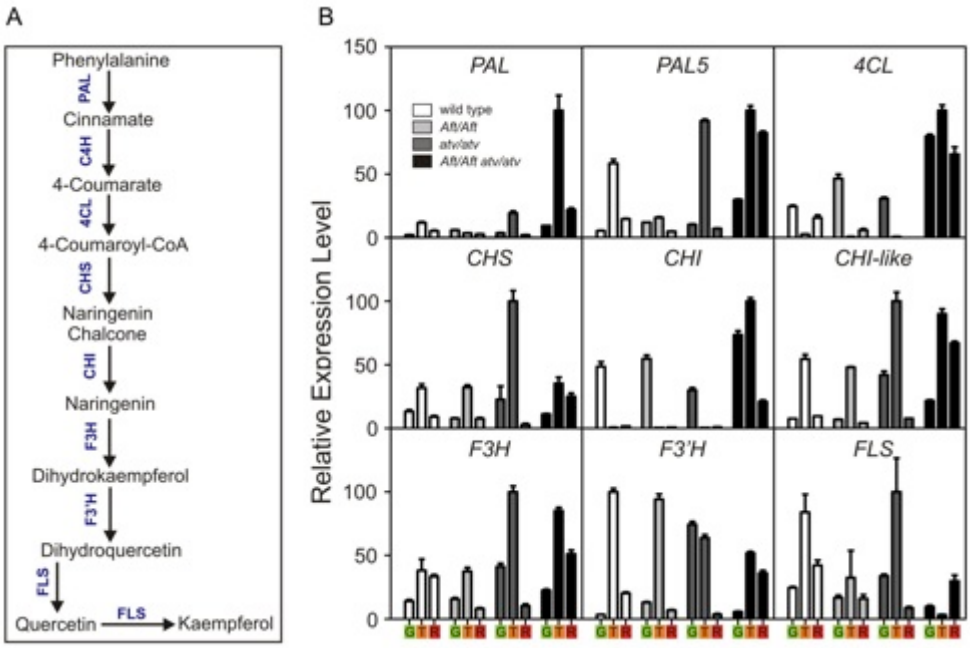

Figure 3. Analysis of gene expression in the early steps of the anthocyanin biosynthetic pathway in the peel of tomato fruits.

(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'-hydroxilase (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 ±SD.

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 (Figure 3B). 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 (Figure 3B). *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*) (Figure 3B). It seems therefore 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 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* (Figure 3B). *PAL* and *PAL5* were strongly expressed, especially at the T and R maturity stages (Figure 3B). *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 was 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-type represent the major class of anthocyanins observed in tomato (Bovy et al., 2007). They are formed by the concerted action of flavonoid 3'5'-hydroxylase (F3'5'H), dihydroflavonol reductase (DFR), and leucoanthocyanidin dioxygenase (ANS) (Figure 4A). Modification with hydroxyl, methyl, glycosyl and acyl groups by the action of specific enzymes, results in the final different anthocyanin structures (Figure 4A). 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) (Figure 4A). The genes encoding for these proteins were therefore all 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 (Figure 4B). 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 (Figure 4B). A very slight induction of some of these genes was also observed in *Aft/Aft* green fruits, whereas *atv/atv* expression patterns were not different from those observed in AC, where a negligible expression of these genes was found (Figure 4B). 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 (Figure 4B). 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 (Figure 4A) and are considered as catalyzing limiting steps in the tomato anthocyanin biosynthetic pathway (Bovy et al., 2007). These data (Figure 4B) revealed a set of genes that are likely to be responsible for the unusual accumulation of anthocyanin in the *Aft/Aft atv/atv* genotype.

Analyzing 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, show indeed an up-regulation in the double mutant which is much higher than in the corresponding single mutant parental line (Figs. 3B and 4B).

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

Anthocyanin metabolism may be tightly interconnected with other important physiological processes. According to this, 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, *SlAN2* and *SlANT1*, were predominantly expressed in the peel of green fruits (Figure 1D), thus suggesting an early effect of these genes on the activation of the anthocyanin pathway. For *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 (Figure 3B), it is likely that also the *Atv* gene is early expressed. 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 (Figure 1C). For all these reasons, RNA from the green fruit peel was used for the transcriptome analysis.

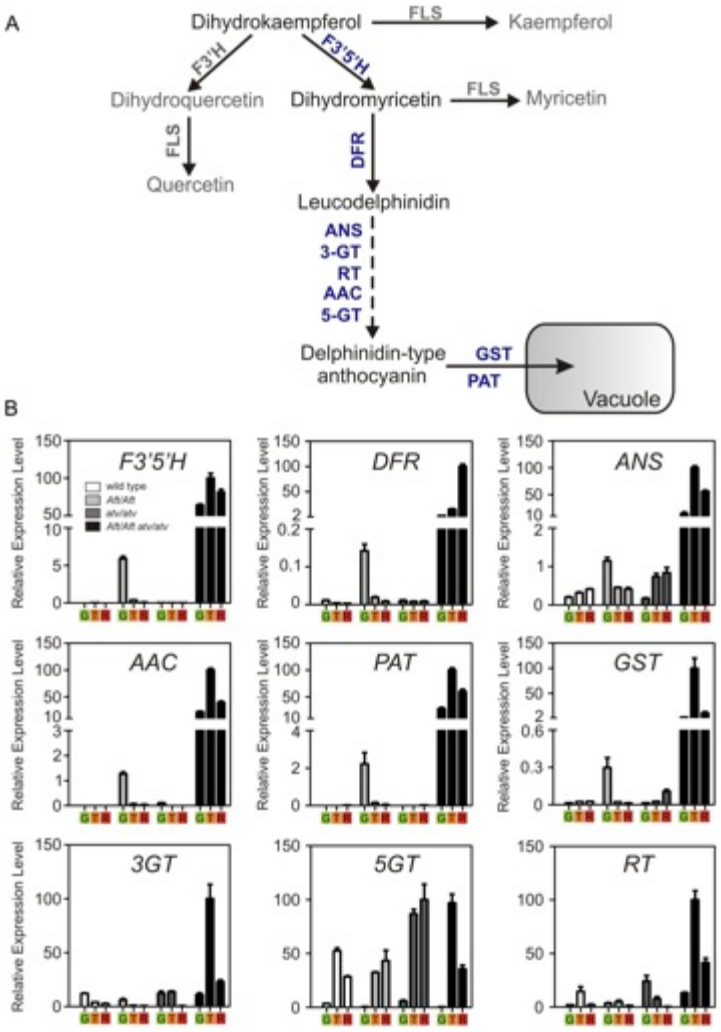

Figure 4. Analysis of gene expression in the late steps of the anthocyanin biosynthetic pathway in the peel of tomato fruits.

(A) Late steps in the anthocyanin biosynthetic pathway. Enzyme names were abbreviated as follows: flavonoid 3'-hydroxilase (F3'H), flavonols synthase (FLS), flavonoid 3'5'-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 ±SD.

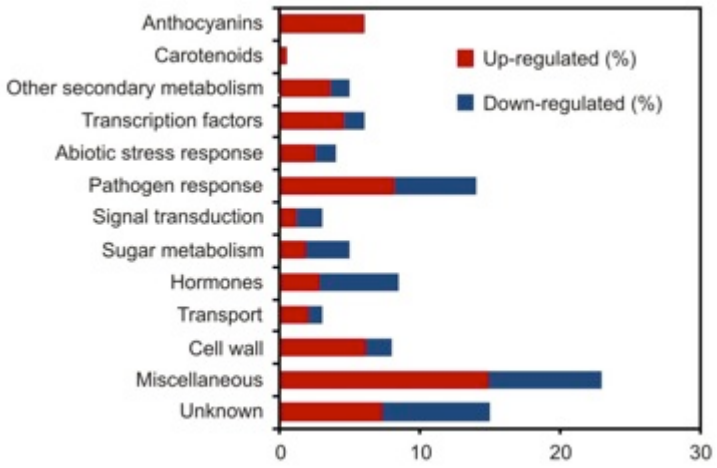

Figure 5. Functional categories of the peel-associated transcripts displaying differential expressions when comparing AC with *Aft/Aft atv/atv* fruits. The distribution of categories is given as a percentage of the total 214 differentially expressed transcripts ($p \leq 0.001$).

We identified 214 differentially expressed genes (DEGs) (p-value ≤ 0.001) in *Aft/Aft atv/atv* fruit peel compared to AC (131 up-regulated and 83 down-regulated). We cannot exclude that some alterations in the expression pattern might be due to the lack of isogenicity between AC and the double mutant line (see Material and methods). However, the identical growth conditions and the high similarity in fruit size and maturation time between the two genotypes allow 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 (Figure 5). All the transcripts of 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), 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. This may be a consequence of the accumulation of anthocyanins, since these compounds represent signaling molecules for the activation of defence 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 defence programmes. 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 defence programme.

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*. 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 (Figure 5). This may lead to hypothesize 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* was 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 till now (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 appears to be very interesting. 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 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.

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 (Figure 5).

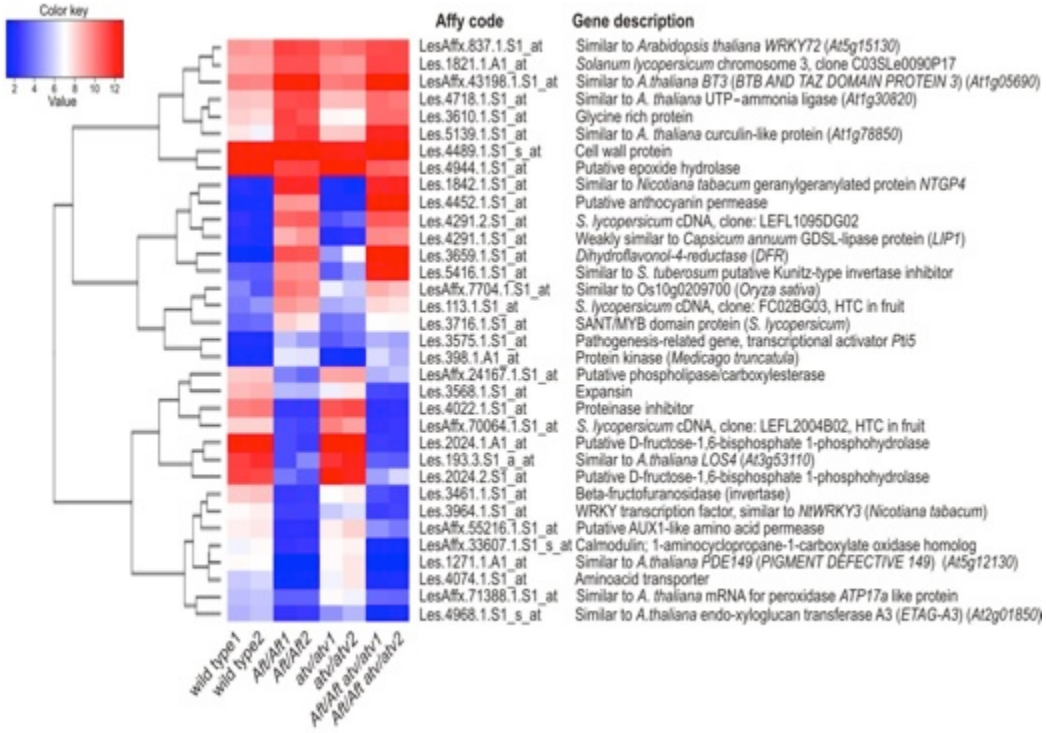

Figure 6. Heatmap of differentially expressed genes showing a common expression pattern in *Aft/Aft atv/atv* and *Aft/Aft* fruit peel. Two different biological replicates for each genotype (indicated as 1 and 2) were used for this experiment.

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*.

As expected from qPCR analyses (Figure 4B), *Aft*-associated DEGs (Figure 6; Tables 1 and 2) include transcripts involved in anthocyanin biosynthesis, such as *PAT* and *DFR*. Both these genes were expressed in *Aft/Aft* and *Aft/Aft atv/atv* at a much higher level than in AC and *atv/atv* (Figure 6). However, their expression in the double mutant was considerably higher than in *Aft/Aft* (Figure 4B). 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 *SlAN2* or *SlANT1* 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) (Figure 6; Table 1 and 2). Furthermore, a considerable number of genes putatively involved in defence 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 these genotypes (Figure 6; Table S3). Besides their metabolic role, plant protease or invertase inhibitors have been found to be involved in plant defence 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 WRKY72*, involved in plant defence and elicitation by chitin and pathogens (Libault et al., 2007), was also commonly up-regulated in* Aft/Aft *and* Aft/Aft atv/atv *(Figure 6; Table S3). A* GDSL lipase *transcript (Les.4291.1.S1_at) encoding a protein similar to* Capsicum annum *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 *(Figure 6; 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 defence.*

Table 1. List of differentially expressed genes showing common up-regulation in *Aft/Aft* and *Aft/Aft atv/atv* with respect to the wild type Ailsa Craig (AC) and *atv/atv*.

Affy_Probe	Probe annotations	Mean AC	Mean *Aft/Aft*	Mean *atv/atv*	Mean *Aft/Aft atv/atv*
Les.4489.1.S1_s_at	Cell wall protein	6217,546	35199,969	4774,267	43024,217
Les.3659.1.S1_at	*Dihydroflavonol-4-reductase* (*DFR*) (*Dihydrokaempferol 4-reductase*)	2,843	1935,490	82,221	17741,600
LesAffx.43198.1.S1_at	*Solanum lycopersicum* chromosome 2 clone C02HBa0146O16. Similar to *Arabidopsis thaliana BT3* (*BTB AND TAZ DOMAIN PROTEIN 3*) (*AT1G05690)*	889,553	6553,600	1070,252	9980,995
Les.4452.1.S1_at	Putative anthocyanin permease	3,503	675,542	3,933	8990,123
Les.5416.1.S1_at	Similar to *Solanum tuberosum* putative Kunitz-type tuber invertase inhibitor	10,399	774,058	20,991	6085,865
Les.5139.1.S1_at	Similar to *Arabidopsis thaliana* curculin-like (mannose-binding) lectin family protein (*AT1G78850*).	150,776	2679,827	306,932	4205,057
Les.1842.1.S1_at	Similar to *Nicotiana tabacum* geranylgeranylated protein *NTGP4*	4,221	3746,291	3,540	4019,411
Les.1821.1.A1_at	*Solanum lycopersicum* chromosome 3 clone C03SLe0090P17	517,186	2025,135	683,567	2313,318
LesAffx.837.1.S1_at	Similar to *Arabidopsis thaliana WRKY72* (*AT5G15130*)	648,195	2447,930	1212,934	2235,764
Les.4291.2.S1_at	*Solanum lycopersicum* cDNA, clone: LEFL1095DG02	4,655	1542,078	10,475	1733,017
Les.3610.1.S1_at	Glycine rich protein	260,281	2190,402	147,182	1281,982
Les.4718.1.S1_at	Similar to *Arabidopsis thaliana* CTP synthase, putative / UTP--ammonia ligase, putative (*AT1G30820*)	338,123	2225,889	379,075	1194,692
Les.4291.1.S1_at	*Solanum lycopersicum* chromosome 2 clone C02SLe0016P15. Weakly similar to *Capsicum annuum* GDSL-lipase protein (*LIP1*)	4,195	568,361	4,765	792,655
LesAffx.7704.1.S1_at	Similar to Os10g0209700 (*Oryza sativa*)	14,212	999,355	77,342	397,907
Les.113.1.S1_at	*Solanum lycopersicum* cDNA, clone: FC02BG03, HTC in fruit	20,051	598,637	38,714	230,907
Les.3716.1.S1_at	SANT/MYB domain protein (*Solanum lycopersicum*)	12,375	243,251	16,283	134,219
Les.398.1.A1_at	Similar to a protein kinase (*Medicago truncatula*)	3,568	84,640	3,283	55,001

Les.3575.1.S1_at	Pathogenesis-related gene, transcriptional activator *Pti5*	4,195	37,333	11,886	33,854

Table 2. List of differentially expressed genes showing common down-regulation in *Aft/Aft* and *Aft/Aft atv/atv* with respect to the wild type Ailsa Craig (AC) and *atv/atv*.

Affy_Probe	Probe annotations	Mean AC	Mean *Aft/Aft*	Mean *atv/atv*	Mean *Aft/Aft atv/atv*
Les.4944.1.S1_at	Putative epoxide hydrolase	7301,199	2314,233	7015,074	1483,297
Les.2024.2.S1_at	Putative D-fructose-1,6-bisphosphate 1-phosphohydrolase	2467,323	12,623	5560,958	48,886
LesAffx.24167.1.S1_at	Putative *Arabidopsis thaliana* phospholipase/carboxylesterase family protein (*AT1G52700*)	312,473	15,069	566,443	44,516
LesAffx.55216.1.S1_at	Putative AUX1-like amino acid permease	186,308	5,114	215,564	19,967
LesAffx.71388.1.S1_at	Similar to *Arabidopsis thaliana* mRNA for peroxidase *ATP17a* like protein	49,187	11,005	123,550	11,941
Les.193.3.S1_a_at	Similar to *Arabidopsis thaliana LOS4* (*LOW EXPRESSION OF OSMOTICALLY RESPONSIVE GENES 4*) (*AT3g53110*)	3083,782	14,244	3922,659	10,616
Les.3461.1.S1_at	Beta-fructofuranosidase (invertase)	324,844	5,940	153,058	7,501
Les.3568.1.S1_at	Expansin	417,478	42,103	145,192	7,401
Les.3964.1.S1_at	WRKY transcription factor, similar to *NtWRKY3* (*Nicotiana tabacum*)	157,314	6,652	65,907	6,636
Les.2024.1.A1_at	Putative D-fructose-1,6-bisphosphate 1-phosphohydrolase	6237,046	6,948	11628,916	5,830
LesAffx.70064.1.S1_at	*Solanum lycopersicum* cDNA, clone: LEFL2004B02, HTC in fruit	270,123	7,472	742,114	5,585
Les.4022.1.S1_at	Proteinase inhibitor	1020,605	5,836	2049,327	5,274
Les.4074.1.S1_at	Aminoacid transporter	61,643	2,992	152,875	3,852
LesAffx.33607.1.S1_s_at	Calmodulin	122,184	5,609	151,232	3,364
Les.4968.1.S1_s_at	Similar to *Arabidopsis thaliana* endo-xyloglucan transferase A3 (*ETAG-A3*) (AT2G01850)	43,500	5,652	31,536	3,304

Les.1271.1.A1_at	Similar to *Arabidopsis thaliana PDE149* (*PIGMENT DEFECTIVE 149*) (*AT5G12130*)	139,012	2,429	169,498	2,334

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 7; Tables 3 and 4) 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* (Figure 7; Table 3), in agreement with the qPCR data (Figure 3B). 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 (Figure 7, Table 3). Moreover, one of the commonly up-regulated genes in *atv* and double mutant was a putative *UDP rhamnose: anthocyanidin-3-glucoside*

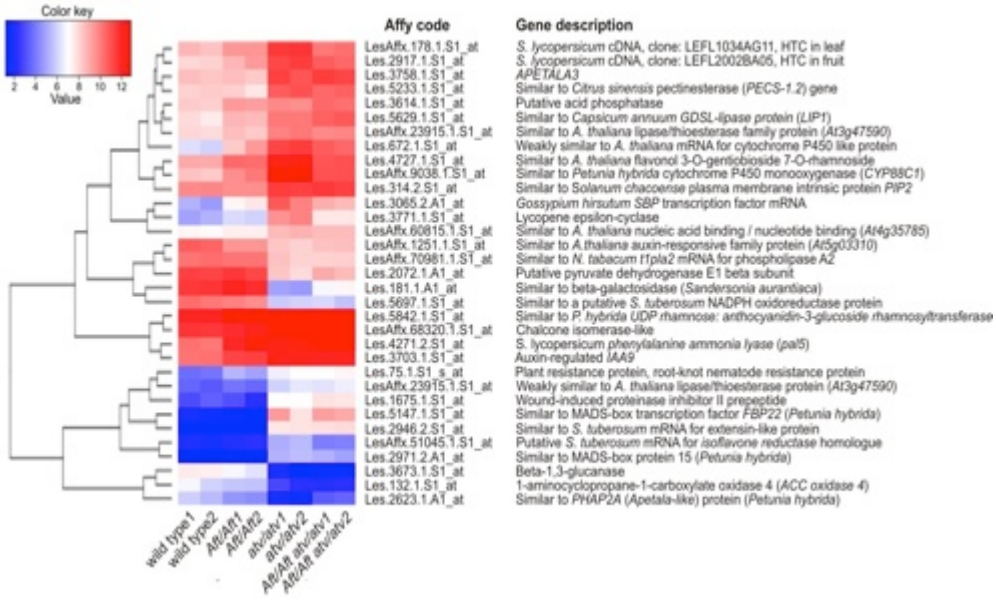

Figure 7. Heatmap of differentially expressed genes showing a common expression pattern in *Aft/Aft atv/atv* and *atv/atv* fruit peel. Two different biological replicates for each genotype (indicated as 1 and 2) were used for this experiment.

rhamnosyltransferase (Les.5842.1.S1_at), possibly involved in anthocyanin glycosylation (Fig 7; Table 3). This data mirrors 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 (Figure 4B). However, since this gene was found expressed also in AC tomatoes in the T stage (Figure 4B), 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* (Figures. 4B and

7; Table 3), 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 (Figure 7, Table 3). Genes coding for a wound-induced proteinase inhibitor (Les.1675.1.S1_at)

Table 3. List of differentially expressed genes showing common up-regulation in *atv/atv* and *Aft/Aft atv/atv* with respect to the wild type Ailsa Craig (AC) and *Aft/Aft.*

Affy_Probe	Probe annotations	Mean AC	Mean *Aft/Aft*	Mean *atv/atv*	Mean *Aft/Aft atv/atv*
Les.5842.1.S1_at	Similar to *Petunia hybrida* mRNA for UDP rhamnose: anthocyanidin-3-glucoside rhamnosyltransferase	4732,237	9494,870	31866,16	21082,556
Les.4271.2.S1_at	*Lycopersicon esculentum* phenylalanine ammonia lyase (*pal5*) gene	1368,610	5600,505	6139,084	14428,954
Les.3650.1.S1_at	*Lycopersicon esculentum chalcone synthase 1* (*CHS1*)	36,023	2081,443	22394, 49	14447,058
LesAffx.68320.1.S1_at	Chalcone isomerase-like	3544,908	4537,280	17233,818	11689,914
Les.3703.1.S1_at	Auxin-regulated *IAA9*	1409,060	2901,120	5254,857	4885,811
Les.314.2.S1_at	Similar to *Solanum chacoense* plasma membrane intrinsic protein *PIP2*	1025,750	937,341	3163,542	3171,549
Les.3758.1.S1_at	*APETALA3*	424,115	417,173	2582,528	3014,262
LesAffx.9038.1.S1_at	Weakly similar *Petunia hybrida* cytochrome P450 monooxygenase (*CYP88C1*); aquaporin-like protein	404,095	1111,610	8826,345	2307,219
Les.4727.1.S1_at	*Solanum lycopersicum* cDNA, clone: LEFL2004G12, HTC in fruit; Similar to an *Arabidopsis thaliana* genomic region affecting the accumulation of flavonol 3-O-gentiobioside 7-O-rhamnoside (Locus AM74803 6)	610,304	1561,677	5715,764	2265,717
Les.5629.1.S1_at	Similar to *Capsicum annuum* GDSL-lipase protein (*LIP1*)	262,348	499,071	1237,261	1940,797
Les.672.1.S1_at	Weakly similar to *Arabidopsis thaliana* mRNA for cytochrome P450 like protein (LOCUS *AK226339*)	88,567	578,988	3018,392	1808,302
Les.2917.1.S1_at	*Solanum lycopersicum* cDNA, clone: LEFL2002BA05, HTC in fruit	321,586	653,964	2885,048	1775,071
Les.5233.1.S1_at	Similar to *Citrus sinensis* pectinesterase (*PECS-1.2*) gene	350,387	307,292	2139,201	1731,669
Les.3614.1.S1_at	Putative acid phosphatase	358,043	760,702	961,996	1525,789
LesAffx.178.1.S1_at	*Solanum lycopersicum* cDNA, clone: LEFL1034AG11, HTC in leaf	455,635	842,940	3534,758	1342,490

LesAffx.23915.1.S1_at	Similar to *Arabidopsis thaliana* esterase/lipase/thioesterase family protein (*AT3G47590*)	294,757	419,317	1228,473	1034,149
Les.5147.1.S1_at	Similar to MADS-box transcription factor *FBP22* (*Petunia hybrida*)	4,600	3,904	523,765	672,149
Les.3065.2.A1_at	*Gossypium hirsutum* SBP transcription factor mRNA, complete cds; Solanum lycopersicum cDNA, clone: LEFL2003DG08, HTC in fruit; putative squamosa promoter-binding protein (*Betula platyphylla*)	41,650	217,929	1598,090	526,371
LesAffx.60815.1.S1_at	Similar to *Arabidopsis thaliana* nucleic acid binding / nucleotide binding (AT4G35785); transformer-SR ribonucleoprotein (*Nicotiana tabacum*)	185,555	220,444	507,039	478,510
Les.2946.2.S1_at	Similar to *Solanum tuberosum* mRNA for extensin-like protein	3,797	3,622	253,448	284,864
Les.1675.1.S1_at	Wound-induced proteinase inhibitor II prepeptide	12,871	9,211	159,688	251,151
Les.3771.1.S1_at	Lycopene epsilon-cyclase	45,624	87,509	1011,259	238,642
Les.75.1.S1_s_at	Plant resistance protein, root-knot nematode resistance protein	18,548	46,252	147,742	178,397
LesAffx.23915.1.A1_at	Weakly similar *Arabidopsis thaliana* esterase/lipase/thioesterase family protein (*AT1G29840*)	14,170	18,197	97,602	114,291
Les.2971.2.A1_at	MADS-box protein 15 (*Petunia hybrida*); also similar to *Solanum tuberosum* proteinase inhibitor 1 PPI3A2 (*PPI3A2*)	3,407	3,499	55,221	43,981
LesAffx.51045.1.S1_at	Similar to a putative *Solanum tuberosum* mRNA for *isoflavone reductase*	5,230	6,346	52,147	22,839

Table 4. List of differentially expressed genes showing common down-regulation in *atv/atv* and *Aft/Aft atv/atv* with respect to the wild type Ailsa Craig (AC) and *Aft/Aft.*

Affy_Probe	Probe annotations	Mean AC	Mean *Aft/Aft*	Mean *atv/atv*	Mean *Aft/Aft atv/atv*
Les.2072.1.A1_at	*Solanum lycopersicum* cDNA, clone: LEFL2012I07, HTC in fruit; putative pyruvate dehydrogenase E1 beta subunit	2951,573	3035,446	341,481	552,311
LesAffx.1251.1.S1_at	Similar to an *Arabidopsis thaliana* auxin-responsive family protein (*AT5G03310*)	1852,794	831,471	372,940	436,410
LesAffx.70981.1.S1_at	Similar to *Nicotiana tabacum t1pla2* mRNA for phospholipase A2	1417,112	1364,979	375,816	343,926
Les.181.1.A1_at	*Solanum lycopersicum* chromosome 2 clone C02HBa0090O01; similar to beta-galactosidase (*Sandersonia aurantiaca*)	3543,005	4414,858	37,916	168,937
Les.5697.1.S1_at	Putative *Solanum tuberosum* NADPH quinone oxidoreductase-like protein	1394,130	1018,799	77,084	73,742
Les.2623.1.A1_at	*Solanum lycopersicum* cDNA, clone: LEFL1010BC06, HTC in leaf; similar to *PHAP2A* (*Apetala-like*) protein (*Petunia hybrida*)	65,797	31,325	4,595	14,836
Les.132.1.S1_at	1-aminocyclopropane-1-carboxylate oxidase 4 (*ACC oxidase 4*), ethylene forming enzyme	112,431	64,835	8,224	6,285
Les.3673.1.S1_at	Beta-1,3-glucanase	219,818	122,185	4,322	4,072

and a putative plant resistance protein (Les.75.1.S1_s_at), both involved in plant defence, 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* (Figure 7, Table 3). 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 (Figure 3B), could be also crucial (Lee et al., 1992).

To summarise, genes involved in important steps of flavonoid biosynthesis, cell wall metabolism and defence pathways, different from the ones found in the previous paragraph, resulted 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. Neither in *Aft* nor in *atv* fruits a similar process could be observed, but only a partial activation of some steps of the pathway. Our data indicate that *Aft* mainly affects the expression of genes directly involved in the anthocyanin production, such as *DFR* and other LBGs. This supports the hypothesis that *Aft* may represent an allele of *SlAN2* or *SlANT1*, which encode MYB transcription factors highly expressed in anthocyanin-accumulating fruits (Figure 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 *SlAN2* and/or *SlANT1* in *Aft* green fruits could be responsible for the activation of the late steps of the biosynthetic pathway (Figs. 1D and 4B), leading to the final limited anthocyanin production (Figure 1A, B, C). On the other hand, qPCR and microarray data indicate that the *atv* mutation might prevalently influence the expression of some of the EBGs involved in the biosynthesis of flavonoid precursors in the fruit (Figs. 3B and 7). This would be in accordance with the hypothesis of a role of this gene in the photomorphogenesis and in the phytochrome signalling 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 (Figure 3B), but also, and even more strikingly, most of the LBGs (Figure 4B). It is therefore likely that *atv* gene synergistically acts with *Aft* on LBGs transcription. For example, a combined effect of transcriptional activation, sustained by the high expression of *SlAN2* and/or *SlANT1*, 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 play 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. However, we cannot exclude that some differentially expressed transcript can be due to a comparison of genetically different lines. 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 (Figures 6 and 7). 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 on-going to verify if 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.

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 done considering that, unlike the mutant lines selected for this study, AC does not produce anthocyanins in the fruit skin. We considered also 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 these characteristics are quite similar between AC and the mutant lines, therefore putting 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-NO_3^-, 1.3 P-PO_4^-, 8 K^+, 4 Ca^{2+}, 1.2 Mg^{2+}, 9 Na^+, 1.5 S-SO_4^{2-}. 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 stem (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, 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 µg 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 5. *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 done using a quantile method (Bolstad et al., 2003). To reduce the number of non-informative genes, we applied an interquantile filter (interquantile range, IQR=0.25). To identify a statistically reliable number of differentially expressed genes among the genotypes, a linear model was performed (Wettenhall and Smyth, 2004). To assess a 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 no. GSE19792; http://www.ncbi.nlm.nih.gov/projects/geo).

Table 5. List of primers used in quantitative RT-PCR analyses.

Gene Name	Gene Description	GenBank ccession number	Forward primer	Reverse primer	TaqMan probe
EF1A	*Elongation factor1-alpha*	X14449	TGCTTGCTTTCACCCTTGGT	CGATTTCATCATACCTAGCCTTGGA	CTGCTGTAACAAGATGGATGC
ANT1	*Anthocyanin 1*	DD030645	AAGTGGATCTCATTTTGAGGCTTCA	TCCTTCCGGGAAGTCTACCA	CAACAGATGGTCACTTATTG
AN2	*Anthocyanin 2*	FJ744761	TTCCAGGAAGGACAGCAAAC	AACGAGGACGAGAATGAGGA	
*PAL**	*Phenylalanine ammonia-lyase*	M83314	ATTGGGAAATGGCTGCTGATT	TCAACATTTGCAATGGATGCA	
PAL5	*Phenylalanine ammonia-lyase 5*	M90692	GGAATTGCAGGGTTGCCACTTT	AAGGCCGCGTTGCCTAAAGAAG	
4CL	*4-coumarate-CoA ligase*	AK323545	GCATTGGAGAATGGTGTGAA	CTCATCGGCCTGAATCAACT	
*CHS**	*Chalcone synthase 1*	X55194	TGGTCACCGTGGAGGAGTATC	GATCGTAGCTGGACCCTCTGC	
*CHI**	*Chalcone isomerase*	AK325510	GTTTTTCACAAACCAACAGTTCTGAT	GAAGCAGTGCTCGATTCCATAAT	
CHI-like	*Chalcone isomerase like*	AK328644	TGAGGCTATTGTGAATGCTCCAG	TAGCACTCTCTAGCTGCACACC	
*F3H**	*Flavanone-3-hydroxylase*	AK329343	CACACCGATCCAGGAACCAT	GCCCACCAACTTGGTCTTGTA	
*F3'H**	*Flavanone-3'-hydroxylase*	AC238569	GCACCACGAATGCACTTGC	CGTTAGTACCGTCGGCGAAT	
*FLS**	*Flavonol synthase*	AK325947	GAGCATGAAGTTGGGCCAAT	TGGTGGGTTGGCCTCATTAA	
*F3'5'H**	*Flavonoid 3'5'-hydroxylase*	DB723744	GGCAATTGGACGAGATCCTG	AAGGAACCTCTCGGGAGTGAA	
DFR	*Dihydroflavonol reductase*	Z18277	CAAGGCAGAGGGAAGATTCATTTG	GCACCATCTTAGCCACATCGTA	ATCCCATCATGCTATCATC
*ANS**	*Leucoanthocyanidin dioxygenase*	AJ785263	GAACTAGCACTTGGCGTCGAA	TTGCAAGCCAGGCACCATA	
AAC	*Anthocyanin acyltransferase*	EU979541	CCCTCCAGTACCACCAGAAA	TTCAGACAACCTTCCAGCAA	
PAT	*Anthocyanin permease*	AY348872	CGGTGTTTCAGTCCCTCCTA	TTGCATCTCCTTGCTGTTTG	
GST	*Glutathione S-transferase*	AC226509	TGGGACACAACAGTGATTTGA	TGGCTTAGATCGGCTAAGGA	
3GT	*Flavonoid: 3-O-glucosyltransferase*	BP893263	GCACATAAGAGTGTTGGCGTTT	TTTCCAAACACTTTCCACCA	
5GT	*Anthocyanin 5-O glucosyltransferase*	CD003048	GTGGCATTTCCTCATTGGAC	TCATCACTCTCAACCACACCA	
RT	*UDP rhamnose: anthocyanidin-3-glucoside*	BP890816	ATGTTGCCACAGAAAGGTGA	CCATCATCACCATCTCCACA	

	rhamnosyltransferase				

*Primer sequences taken from Bovy *et al.* (2002).

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