Molecular Aspects of Anthocyanin fruit Tomato in Relation to high pigment-1

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Abstract

The tomato Anthocyanin fruit (Aft) genotype is characterized by purple color in skin and outer pericarp of its fruits due to higher levels of anthocyanins—flavonoid metabolites. Our objectives were to carry out metabolic and molecular characterization of this genotype, emphasizing its interaction with the high pigment-1 (hp-1) mutation, known to increase flavonoids in tomato fruits. These objectives fit the growing interest in developing tomato fruits with higher levels of functional metabolites. Our results show that 1) Aft fruits are also characterized by significantly higher levels of the flavonols quercetin and kaempferol, thus enhancing their functional value; 2) the tomato Anthocyanin1 (Ant1) gene, encoding a Myb transcription factor, displayed nucleotide and amino acid polymorphisms between the Aft genotype and cultivated genotypes; 3) a DNA marker based on Ant1 showed that the Aft trait is encoded by a single locus on chromosome 10 fully associated with Ant1; and 4) double homozygotes Aft/Aft hp-1/hp-1 plants displayed a more-than-additive effect on the production of fruit anthocyanidins and flavonols. This effect was manifested by approximately 5-, 19-, and 33-fold increase of petunidin, malvidin, and delphinidin, respectively, in the double mutants compared with the cumulative levels of their parental lines.

Flavonoids are polyphenolic compounds that occur naturally in most plants. Flavonoids are present in fruits, vegetables, and beverages derived from plants and in dietary supplements or herbal remedies. Based on their core structure, the aglycone, they can be grouped into different classes, such as chalcones, flavanones, dihydroflavonols, flavonols, and anthocyanins. As a group, flavonoids are involved in many aspects of plant growth and development, such as pathogen resistance, pigment production, ultraviolet light protection, pollen growth, and seed coat development (Bovy et al. 2002).

There is increasing evidence suggesting that flavonoids are health-promoting components in the human diet as a result of their high antioxidant capacity and their ability, in vitro, to induce human protective enzyme (recently summarized by Bovy et al. 2002; Jones et al. 2003; Levin et al. 2006). Based on studies of this type, there is growing interest in the development of food crops enriched with health-protective flavonoids. An excellent candidate for such an approach is tomato (Solanum lycopersicum L.), one of the most important food crops worldwide (Bovy et al. 2002; Willits et al. 2005). Efforts have been therefore invested in increasing the flavonoid content in the tomato fruit (Bovy et al. 2002; Verhoeyen et al. 2002; Levin et al. 2006).

Overexpression of structural genes involved in the flavonoid biosynthetic pathway resulted in transgenic tomato lines with significantly altered flavonoid content (Muir et al. 2001; Colliver et al. 2002; Verhoeyen et al. 2002). Most notably, 1) an up to 78-fold increase in total fruit-peel flavonols was achieved through ectopic expression of chalcone isomerase (Muir et al. 2001), 2) chalcone synthase and flavan-3-ol synthase transgenes were found to act synergistically and significantly upregulate flavonol biosynthesis in flesh tissue (Colliver et al. 2002), and 3) transgenic tomato plants accumulating new flavonoid compounds in their fruit peel were engineered using structural flavonoid genes from different plant sources, thus enhancing their total antioxidant capacity (Schijlen et al. 2006). An overall increase in flavonoid levels in tomato fruit was also achieved by simultaneous overexpression of the maize genes Lc and C1, encoding Myc- and Myb-type transcription factors, respectively (Bovy et al. 2002). Further, T-DNA activation-tagging experiments in tomato identified a Myb transcriptional regulator of anthocyanin biosynthesis, termed Anthocyanin1 (Ant1), which shares high homology with Petunia An2 (Mathews et al. 2003). These mutant ant1 tomato plants yielded fruits with purple spotting on the epidermis that could be observed at x66 magnification.

Despite the relative success obtained in increasing flavonoid content in tomato fruits by transgenic modifications, there is an ongoing interest in breeding a high...
flavonoid tomato without genetic engineering (Willits et al. 2005). This interest is motivated by customers’ reluctance to consume transgenic fruits and vegetables.

As recently summarized (Jones et al. 2003), fruits of several tomato species closely related to the cultivated tomato contain significantly higher amounts of anthocyanins (Rick 1964; Giorgiev 1972; Rick et al. 1994). The Anthocyanin fruit (Aft, formerly Aft) from Solanum chilense, Aubergine (Aub) from Solanum hyptersioides, and the recessive atrovirulacum (avt) mutation from Solanum cheesmaniae cause anthocyanin expression in tomato fruit. Recently, the wild species Solanum pennelli v. paterulum was also shown to be a source for enriching tomato fruits with flavonoids (Willits et al. 2005).

The Aft phenotype is considered to be controlled by a single dominant gene (Giorgiev 1972; Jones et al. 2003). However, the allelic relationships between Aft and other genes increasing fruit anthocyanin content, in particular Aub, are unclear. Aub was mapped to the tomato chromosome 10 (Rick et al. 1994), and it was later suggested that it lies in the chromosomal inversion identified between S. hyptersioides and S. hyptersicum on this chromosome (Pertuze et al. 2002). Stable homozygotes of Aub have not been obtained (Jones et al. 2003). This instability that has impeded traditional allele tests with Aft, S. chilense, the donor genome for Aft, is not known to contain any chromosomal inversions in relation to S. hyptersicum. Nonetheless, it was hypothesized that Aft is an allele of Aub and resides on chromosome 10. However, 6 restriction fragment length polymorphism (RFLP) loci surveyed along chromosome 10 of Aft mutant plants failed to uncover an S. chilense introgression (Jones et al. 2003).

Another approach to increase fruit flavonoids is through the introgression of high pigment (hp) mutations. Tomato hp mutations (hp-1, hp-1w, hp-2, hp-2j, and hp-2dg) are best known for their positive effect on carotenoid levels in ripe-red fruits (Mochizuki and Kamimura 1984; Wann et al. 1985; Levin et al. 2004), yielding plants with poor horticultural performances in comparison to its allelic hp-1 mutation. Thus, it would be of practical importance to also analyze the interaction between Aft and hp-1 mutants.

This study was designed to 1) characterize fruits harvested from Aft plants emphasizing other flavonoids accumulating in the mature fruits in addition to anthocyanins, 2) molecularly tag the gene locus encoding the Aft mutant phenotype in order to exploit its nucleotide sequence as a DNA marker to expedite breeding, and 3) quantitatively characterize the interaction between the Aft locus and hp-1.

## Materials and Methods

### Plant Material, Crosses, and Growth Conditions

Accession LA1996 containing Aft was obtained from the C. M. Rick Tomato Genetics Resource Center, Davis, CA. Moneymaker, a red-fruited open-pollinated fresh-market type tomato, was provided by R. E. Kendrick and M. Koornneef (Wageningen Agriculture University, the Netherlands). Seeds from the red-fruited open-pollinated tomato cv. Ailsa Craig and a near-isogenic line homozygous for the hp-1 mutation were provided by J. J. Giovannoni of the Boyce Thompson Institute for Plant Research, Ithaca, NY. Other red-fruited open-pollinated cultivars that were used in this study, VF36 (LA0490) and Rutgers (LA1090), were also obtained from the C. M. Rick Genetics Resource Center.

A cross was made between both cv. Moneymaker and Ailsa Craig hp-1/hp-1 as maternal lines and LA1996 as a paternal line. F1 plants resulting from the latter cross were also allowed to self-pollinate to generate an F2 population segregating for the hp-1 mutation and the 2 Aft alleles: Aft originating from LA1996 and Aft originating from Ailsa Craig. A plant homozygous for the hp-1 mutation and heterozygous at the Aft locus were selected from the above F2 population and allowed to self-pollinate in order to generate an F3 population segregating for the 2 Aft alleles in homozygous hp-1/hp-1 background.

Plants were transplanted and grown at 2 locations in central Israel, at the Volcani Center or on the premises of Gedera Seed Co, Gedera, central Israel. During the summer seasons, plants were grown in the open field and/or in a screen house and during the winter seasons in a controlled heated greenhouse (minimal temperature 18 °C). Transplanting in summer seasons was carried out during the first week of May, whereas in the winter season, transplanting was carried out during the first week of November. Statistical comparisons between purebred lines and F1 hybrids were carried out in a randomized block design (3 blocks with 5 plants of each genotype in each plot). Association studies were carried out in a completely randomized fashion.

### Genomic DNA Extraction

Genomic DNA was extracted from individual plants according to Fulton et al. (1995).

### Design of Polymerase Chain Reaction Primers

All DNA primers used during the course of this study were purchased from the Molecular Biology Center, Ness-Ziona, Israel. Sequence analysis and locus-specific primer design
were carried out with the DNAMAN sequence analysis software v4.1 (Lynnon BioSoft, Quebec, Canada). Primers for real-time polymerase chain reaction (PCR) analysis were designed based on the supplemental material in Bovy et al. (2002) followed by cross-validation with current gene databases (the NCBI [http://www.ncbi.nlm.nih.gov/] and the DFCI Tomato Gene Index [http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=tomato]).

Genotyping hp-1

A pyrosequencing system was used to genotype hp-1 as was earlier described (Lieberman et al. 2004).

Real-Time PCR Analysis

The Aβ phenotype is characterized by irregular purple color in skin and outer pericarp tissues of its fruits (Jones et al. 2003; van Tuinen et al. 2006), initially appearing at the latest mature-green fruit stage. This phenotype is very similar to phenotypes obtained by activation tagging and transformation of the tomato Ant1 gene (Mathews et al., 2003). Mathews et al. (2003) also stated “overexpression of Ant1 resulted in upregulation of genes that encode proteins in the early chalcone synthase (chs) and late dihydroflavonol reductase (dfr) steps of anthocyanin biosynthesis.” Preliminary real-time PCR analyses carried out on Aβ mutants indicated that expression of these genes is highly upregulated in fruit peels taken from fruit regions expressing purple color in comparison to those that remain green (results not shown). Therefore, RNA was extracted from fruit peels of mature-green tomato fruits expressing the highest levels of anthocyanins. When no signs of purple color were visible, samples were taken at random from different fruit regions. Three squares of 1 mm² from each of the fruits harvested in each experiment were analyzed. The RNA extraction was carried out using TRIzol reagent system (Invitrogen Corp., Carlsbad, CA). Possible genomic DNA contaminants were digested with TURBO DNA-free (Ambion Inc., Austin, TX), and the total RNA was then used as the template for cDNA synthesis using the iScript cDNA synthesis kit with random and oligo-dT primers (Bio-Rad Laboratories, Hercules, CA).

The real-time PCR analysis was performed using the SYBER GREEN PCR Master Mix (Applied Biosystems, Foster City, CA): initial incubation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 61 °C for 30 s, and polymerization at 72 °C for 30 s. Throughout this study, 18S ribosomal RNA was used as reference gene and the primers designed for it, as well as for the other genes analyzed—chs, chs2, dfr, and Ant1—were as follows:

chs1: F = 5′-GGCTCTCATAAAAAGAGGCC-TAG-3′, R = 5′-TAAGCCCAGCCCCTAAGC-3′;
chs2: F = 5′-CATCCAAAGAGGCTTAGTACC-3′, R = 5′-TATGGAGCAAGTCTCAAACA-3′;
dfr: F = 5′-TCAACTGAGTTTACATA-3′, R = 5′-AGAACCCGTGAGGAGAATG-3′;
Ant1: F = 5′-GTTCTTGTTGTTGAGATG-3′, R = 5′-GATACGCGAGAGCCTTCAG-3′.

Samples were analyzed in duplicates, using the GeneAmp 5700 Sequence Detection System, and data were collected and analyzed with the GeneAmp 5700 SDS software (Applied Biosystems). The relative abundance of the examined gene transcripts was calculated by the formula: 2^{(CT_{target} - CT_{reference})}, where Ct represents the fractional cycle number at which the fluorescence crosses a fixed threshold (usually set on 0.1).

Genotyping of Ant1 and Structural Genes of the Flavonoid Biosynthetic Pathway

Genotyping was carried out for the purpose of linkage analysis and/or polymorphism detection using PCR followed by restriction endonuclease digestions. The primers used in these PCR amplifications are as follows: Ant1: F = 5′-GAAGAGGAGCTGATGATG-3′, R = 5′-GTTGATGGGTGTTGATTT-3′;
chs1: F = 5′-GATACGCTGAGATG-3′, R = 5′-TAAGCCCATAGCCACTAAGC-3′;
dfr: F = 5′-GATAAGGACTTGGCCC-3′, R = 5′-GATAAGGAGAGGCCCCATTGAG-3′;
f3h: F = 5′-CCATTCAAAGACGAGTTAGCAG-3′, R = 5′-GACAGAAAGGCCCAAGTTAAG-3′. PCR was carried out using initial incubation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and polymerization at 72 °C for 60 s. A final elongation step at 72 °C was carried out for 7 min after the completion of the above cycles. PCR amplification products were visualized by electrophoresis in 1.0% agarose gels stained with ethidium bromide. The DNA size marker used throughout this study is a 2-log DNA size ladder purchased from New England BioLabs (Ipswich, MA).

Anthocyanidin and Flavonol Extraction and Quantification

All chemicals and solvents were purchased from Sigma (St Louis, MO). Samples of fresh tomato skins (0.1–0.3 g) were taken from ripe fruit parts expressing the highest levels of anthocyanin in order to represent potential, not average, flavonoid production. These samples were ground in liquid nitrogen, and the pigments were extracted in the dark with 2 ml of cold methanol:water:acetic acid (11:5:1; Markham and Ofman 1993). Extracts were spun for 10 min at 20 800 g (14 000 rpm), leaving the anthocyanins in the supernatant. Further purifications were with two-third volumes of hexane. Samples were then concentrated to 0.5 ml, hydrolyzed by boiling with equal volumes of methanol and 2 N HCl for 1 h and passed through a 0.45-μm polyvinylidene difluoride filter (Nalgene, Rochester, NY).

Anthocyanidin and flavonoid composition was determined using a high performance liquid chromatography (HPLC) (Shimatzu, Japan) equipped with an LC-10AT pump, an SCL-10A controller, and an SPD-M10AVP photodiode array detector. Extracts were loaded onto an...
Mapping the Ant1 and Aft Genes to the Tomato Genome

The Ant1 gene, found in course of this study to be associated with the Aft locus, was mapped to the tomato genome utilizing S. pennellii introgression lines (Eshed et al. 1992), as was earlier demonstrated (Levin et al. 2000). DNAs extracted from individual plants of each of the introgression lines, including their original parental lines M82 and S. pennellii, were used as templates in PCRs. The primers used for these reactions were (Mathews et al. 2003): F: 5’-TCCCCGGGATGAACAGTACATCTATG-3’ and R: 5’-GGACTAGTTTAATCAAGTAGATTCATAAGTCA-3’. The PCR was carried out using initial incubation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and polymerization at 72 °C for 60 s. A final elongation step at 72 °C was carried out for 7 min after the completion of the above cycles. The PCR products obtained were visualized by ethidium bromide. Restriction endonuclease digestion was not needed in order to obtain polymorphism between the parental lines: M82 (LA3475) and S. pennellii (LA0716).

Sequencing of the Ant1 Gene

The Ant1 gene was PCR amplified from individual plants of the Aft mutant (LA1996) and an S. lycopersicum genotype cv. Ailsa Craig. The resulting products were directly sequenced on both strands in an overlapping manner using primers complementary to the Ant1 gene (F: 5’-TCCCCGAGAAGTTATTGAGGTA-3’ and R: 5’-GGACTAGTTTAATCAAGTAGATTCATAAGTCA-3’). Sequencing was carried out with an ABI PRISM 377 automated DNA sequencer (Applied Biosystems).

Results

Aft Fruits Share Higher Levels of Flavonols in Addition to Anthocyanidins

In a preliminary study carried out in 2 screen houses located on the premises of Gedera Seed Co, during the winter season of 2004/2005, we obtained the initial indication that fruits of the Aft genotype share higher levels of the flavonols quercetin and kaempferol in addition to anthocyanidins when compared with 2 red-fruited genotypes VF36 and Rutgers (results not shown). To confirm these results, the Aft genotype LA1996, red-fruited Moneymaker plants, and their F1 plants were grown in a randomized block design in an open field located at the Volcani Center during the summer season of 2005. Fruits were sampled at the ripe stage and analyzed to determine the levels of flavonols and anthocyanidins in fruit peel. Major anthocyanidins and flavonoids identified in ripe fruits and their average concentrations are presented, according to genotype, in Tables 1 and 2, respectively.

Results demonstrate statistically significant higher levels of the anthocyanidins delphinidin, petunidin, and malvidin in the peel of mature fruits harvested from Aft/Aft plants compared with the red-fruited Moneymaker plants (Table 1), in agreement with an earlier report (Jones et al. 2003). In addition, our results show that fruits of the Aft/Aft mutant plants are also characterized by statistically significant higher levels of the flavonols quercetin and kaempferol (Table 2). Quercetin and kaempferol concentrations were found to be approximately 3.6- and 2.7-fold higher, respectively, in

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Major anthocyanidins detected</th>
<th>Malvidin (mean ± SE)</th>
<th>Delphinidin (mean ± SE)</th>
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</thead>
<tbody>
<tr>
<td>Aft/Aft</td>
<td>Petunidin (mean ± SE)</td>
<td>1652.50 ± 290.2</td>
<td>447.50 ± 91.6</td>
</tr>
<tr>
<td>Aft/+</td>
<td>Malvidin (mean ± SE)</td>
<td>107.20 ± 57.3</td>
<td>27.30 ± 15.8</td>
</tr>
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<td>+/+</td>
<td>Delphinidin (mean ± SE)</td>
<td>0.00 ± 0</td>
<td>0.00 ± 0</td>
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<tr>
<td>P(F)</td>
<td></td>
<td>0.005</td>
<td>0.008</td>
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mature fruits of the Aft/Aft genotype compared with those of red-fruited Moneymaker plants.

Results presented in Tables 1 and 2 show that anthocyanin and flavonol concentrations in the fruit skins of heterozygous F₁ plants are usually higher compared with the red-fruited genotype but much lower than the LA1996 genotype (Aft/Aft). These results indicate a partially dominant effect of the Aft gene. Our statistical analysis, however, failed to reveal statistically significant differences between average anthocyanin and flavonol concentration in fruits obtained from F₁ plants when compared with their red-fruited counterparts. These results demonstrate that the Aft gene effect may be inconsistent and influenced by environmental factors.

**Aft Shows Transcriptional Upregulation of Key Genes of the Flavonoid Pathway**

RNA samples for real-time PCR were extracted from mature-green fruits harvested from LA1996 plants and the 2 red-fruited genotypes, VF36 and Rutgers, planted within the framework of the preliminary experiment mentioned above. After cDNA synthesis and real-time PCR analysis, these 3 genotypes were compared in relation to the transcriptional profile of these 2 genes, PCR amplified the corresponding genomic regions from LA1996 and 2 red-fruited cultivars, VF36 (LA0490) and Rutgers (LA1090), and digested them with 31 restriction endonucleases. No polymorphism was obtained for the dfr and Ant1 genes, a dominant gene implicated in the anthocyanin biosynthesis, that was found to be transcriptionally altered in our studies (see former section). These results, were later confirmed by direct sequencing, agree with our following findings that Aft resides on chromosome 10 because 3 copies were thus far reported for tomato dfr (chromosomes 5, 6, and 9) and a single copy for Ant1 on chromosome 2 as summarized by De Jong et al. (2004). Results mentioned thus far have led us to speculate that a regulatory gene, such as Ant1, may be the gene that controls the Aft phenotype.

**Ant1 Displays Nucleotide and Amino Acid Polymorphisms in Aft Plants**

T-DNA activation-tagging experiments in tomato identified a Myb transcriptional regulator of anthocyanin biosynthesis, termed Ant1, which shares high homology with *Petunia An2* (Mathews et al. 2003). These mutant ant1 tomato plants yielded fruits with purple spotting on the epidermis. Similar to our fruit transcriptional results, ant1 mutant seedlings showed upregulation of genes that encode proteins active at the early (chs1) and later (dfr) stages of anthocyanin biosynthesis (Mathews et al. 2003). The Ant1 gene sequence was later used as a RFLP probe to show a complete cosegregation, using 245 F₂ individuals, between Ant1 and the pepper A gene, a dominant gene implicated in accumulation of anthocyanin pigments in the foliage, flower, and immature fruit (Borovsky et al. 2004). The A gene was mapped to the pepper chromosome 10, a chromosome that was earlier shown to be nonpolymorphic in LA1996 (Jones et al. 1996).
et al. 2003). Nonetheless, we decided to sequence characterize the Ant1 gene from LA1996 and the red-fruited cv. Ailsa Craig plants. Our sequence analysis revealed multiple nucleotide differences between the 2 genotypes in both coding and noncoding regions of the Ant1 gene (Figure 1). Comparison of the amino acid sequence between cv. Ailsa Craig and LA1996 revealed 8 amino acid differences between the 2 genotypes, some of which can be regarded as major differences (Figure 2).

Based on the nucleotide sequence differences between LA1996 and the red-fruited genotypes in the Ant1 gene, we have designed primers to use in PCR. Amplification products were digested with NcoI restriction endonuclease to give codominant polymorphisms between the Ant1 alleles originating from S. lycozierium (cv. Moneymaker, +/+), S. chilense (LA1996, Aft/Aft), and their F1 hybrid (Aft/+), as shown in Figure 3.

The nucleotide and protein sequence polymorphism described above were accompanied by a statistically significant transcriptional downregulation of the Ant1 gene in tomato peel taken from fruits harvested from LA1996 (Aft), as compared with the red-fruited Moneymaker counterparts or F1 plants resulting from the cross between these 2 genotypes (Table 3).

The Ant1 Gene Is Completely Associated with the Aft Locus

We have carried out a linkage analysis study to determine whether Ant1 and the Aft trait are associated. For this purpose, we have generated an F2 population resulting from a cross between LA1996 and cv. Ailsa Craig, homozygous for the hp-1 mutation. We have used the hp-1 hp-1 mutant plants due to earlier results showing a strong increase in flavonoid accumulation in ripe-red fruits harvested from mutant plants (Yen et al. 1997), hypothesizing that exaggeration of flavonoid accumulation may be observed in hp-1 hp-1 mutant plants that also carry the Aft allele. A total of 247 F2 plants were genotyped for both the hp-1 and Ant1. The trait of anthocyanin accumulation was recorded by visual inspection of mature-green and ripe fruits, focusing on the lower parts of the fruit rather than fruit shoulder. Our results showed a strong association between the Ant1C and the trait of anthocyanin accumulation. Nonetheless, 4 heterozygous Ant1C/Ant1L plants failed to show anthocyanin accumulation in the mature-green or ripe fruits as would be expected assuming Ant1C is dominant over Ant1L. Regarded as recombinants, these plants would indicate an approximately 0.8 cM distance between the Ant1 and Aft genes (calculated on F2 basis). We however do not believe these are recombinants because, under our growth conditions, we at times failed to observe a visible phenotype in heterozygous Ant1C/Ant1L plants resulting from the cross between LA1996 and several red-fruited open-pollinated cultivars, including Ailsa Craig. The inability of heterozygous plants to display phenotypes is also well demonstrated in our metabolomics data presented in Tables 1 and 2, showing that the average anthocyanin and flavonol content in fruits harvested from Aft/+ F1 plants are more similar to their homozygous +/- than to their homozygous Aft/Aft counterparts. To further validate our claim of a possible complete linkage between Ant1 and Aft, the 4 heterozygous Ant1C/Ant1L F2 plants that did not display the characteristic Aft phenotype were allowed to self-pollinate. Sixty plants of each of 2 of the resulting F3 populations were planted during the summer season of 2006 and the other 2 during the summer season of 2007 in an open field located at the Volcani Center. Visual inspection of their fruits on ripening revealed that these 4 F3 families indeed segregate for the Aft trait as would be expected from heterozygous plants. In addition, a plant homozygous for the hp-1 mutation, enhancing the Aft phenotype (see herein below), and heterozygous for the Ant1 gene (hp-1 hp-1 Ant1C/Ant1L) was allowed to self-pollinate, and the resulting F3 plants were genotyped for the Ant1 gene. Eighteen plants representing each of the 3 resulting genotypes were planted during the summer season of 2006 in a screen house located at the Volcani Center. On fruit maturation, these plants were visually inspected, and a complete association was found between the Ant1 genotype and the Aft phenotype.

The Aft Locus Maps to the Tomato Chromosome 10

The complete association we have obtained between the Aft gene, introgressed from LA1996, and the Ant1 gene sequence enabled us to map the Aft gene onto the tomato genome. Our results showed that the Ant1 maps to the longer arm of the tomato chromosome 10, exclusively to introgression line 10-3, spanning a region between markers TG63 and TG233 (Figure 4). The association obtained in this study between Ant1 and Aft indicates that the gene that causes the Aft phenotype is also localized to the long arm of the tomato chromosome 10.

The hp-1 Mutation Exaggerates Anthocyanidin and Flavonol Expression of Ant1C

Initial indication that hp-1 can intensify anthocyanidin and flavonol expression, attributed by the Aft genotype, was obtained in our F2 population originating from Ailsa Craig × LA1996 cross (results not presented). However, this population segregated for 2 additional mutations that may affect metabolite levels: 1) sp conferring a determinate plant habit—Ailsa Craig is indeterminate (sp+), whereas LA1996 is determinate (sp), and 2) u conferring a uniform fruit ripening—Ailsa Craig is characterized by a darker green fruit shoulder due to chloroplast accumulation (u+), whereas LA1996 lacks this phenotype and therefore bears a uniform ripening fruits (u). Therefore, a determinate (sp) uniform ripening (u) Ant1C/Ant1C hp-1 hp-1 F2 plant was selected to evaluate the interaction between Ant1 and hp-1 in a more uniform background. This plant was allowed to self-pollinate, and ripe fruits were harvested from 18 plants of each of the resulting F3 genotype groups: Ant1C/Ant1C hp-1 hp-1, Ant1C/Ant1T hp-1 hp-1, and Ant1T/Ant1T hp-1 hp-1, as well as their initial parental lines, Ant1C/Ant1C hp-1 hp-1, Ant1T/Ant1T hp-1 hp-1, and Ant1T/Ant1T hp-1 hp-1.
Figure 1. Nucleotide sequence comparison of the *Ant1* gene between cv. Ailsa Craig (GenBank accession EF433416, upper rows) and LA1996 (GenBank accession EF433417, lower rows). Start and stop codons are underlined in both sequences, and intronic regions are gray shaded.
+/+ (LA1996) and Ant1+/Ant1+ bp-1/bp-1 (cv. Ailsa Craig homozygous for the bp-1 mutation). Results presented in Table 4 show that the composite genotype Ant1+/Ant1+ bp-1/bp-1 displays a significant more-than-additive effect on the anthocyanidins delphinidin, petunidin, and malvidin in comparison to its initial parental lines. This genotype exhibited a similar tendency of increased levels of the flavonols quercetin and kaempferol as displayed in Table 5. Interestingly, the Ailsa Craig genotype, P2(Ant1+/Ant1+ bp-1/bp-1), displayed some anthocyanin accumulation in their fruits, whereas the respective F3 genotypes, F3(Ant1+/Ant1+ bp-1/bp-1), did not. This effect can be attributed to the u+ mutation of the Ailsa Craig genotype.

Discussion

The genetics and biochemistry of anthocyanin biosynthesis and regulation have been studied extensively in model species, such as Petunia, Arabidopsis, and maize (Holton and Cornish 1995; Winkel-Shirley 2001), and recently in cultivated species, such as sweet potato (Mano et al. 2007) and apple (Espley et al. 2007). In the vegetable crops of the Solanaceae family, several mutants impaired in anthocyanin biosynthesis have been known for years, but few of the genes controlling this variation have been cloned. Recently, through genetic mapping in tomato and the use of comparative maps of other Solanaceae species, several pigmentation genes were identified as potential candidates for known color mutants in tomato, potato, pepper, and eggplant (De Jong et al. 2004). In addition, T-DNA activation-tagging experiments in tomato identified a transcriptional regulator of anthocyanin biosynthesis, Ant1, which shares high homology with Petunia An2 (Mathews et al. 2003). This gene was later found to be highly associated to the A locus that accumulates anthocyanin pigments in the foliage, flower, and immature pepper fruits (Borovsky et al. 2004).

The main objectives of this study were to carry out a metabolic as well as a molecular characterization of the Aft mutant and also to characterize the interaction between the Aft locus and the bp-1 mutation, typified by increased fruit flavonoid. These objectives fit the growing interest in developing tomato fruits enriched with such metabolites.

The Aft phenotype was confirmed lately as being encoded by a single dominant gene (Jones et al. 2003). Jones et al. (2003) also reports that 1) anthocyanin concentration, as measured by the pH differential method, of pigment-rich pericarp and skin tissues from the Aft genotype LA1996 was 20.6 mg/100 g and 66.5 mg/100 g, respectively, and 2) fruit of accession LA1996 was found to contain predominantly petunidin, followed by malvidin and delphinidin. However, no results were presented in this report regarding possible increases in other flavonoids.

Our results (Table 1) are in full agreement with the anthocyanin profile of LA1996, reported earlier by Jones et al. (2003). In addition, results presented in Table 2 provide, to our knowledge, the first documentation for significant accumulation of 2 important flavonoids in the Aft genotype: quercetin and kaempferol. These additional properties
emphasize the potential of the \textit{Aft} mutation to further enhance the functional properties of cultivated tomato fruits.

DNA markers associated with traits of economical importance are regarded as excellent tools to expedite breeding, in particular, when such DNA markers, which can be assayed at the very early seedling stages, can mark traits expressed at much later stages of plant development. Fruit traits analyzed in the course of this research are good examples of such traits because they are expressed upon fruit ripening, a late reproductive stage in plant development. In this study, we have demonstrated a full association between the \textit{Ant1} gene sequence and the trait of anthocyanin accumulation \textit{Aft} introgressed from \textit{S. chilense}. \textit{Ant1} can therefore serve as an excellent DNA marker for breeding purposes. Due to semidominant nature of the \textit{Aft} gene, it is suggested that only homozygous \textit{Ant1CAnt1C} plants, comprising only a quarter of an \textit{F} \textit{2} population, should be further inspected.

\textbf{Table 4.} Average relative concentrations of major anthocyanidins detected in ripe fruits of parental and \textit{F} \textit{3} genotypes

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<th>Genotype</th>
<th>Major anthocyanins detected</th>
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<tr>
<td></td>
<td>Petunidin (mean ( \pm ) SE)</td>
<td>Malvidin (mean ( \pm ) SE)</td>
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<td></td>
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<tr>
<td>\textit{P} \textit{1} ((\textit{Ant1C}/\textit{Ant1C} +/+)/\textit{P})</td>
<td>157.5\textsuperscript{C} ( \pm ) 18.5</td>
<td>34.5\textsuperscript{C} ( \pm ) 1.5</td>
<td>10.5\textsuperscript{C} ( \pm ) 3.5</td>
<td></td>
</tr>
<tr>
<td>\textit{P} \textit{2}((\textit{Ant1F}/\textit{Ant1C}/\textit{hp-1}/\textit{hp-1})</td>
<td>943.3\textsuperscript{B} ( \pm ) 856.5</td>
<td>26.0\textsuperscript{C} ( \pm ) 3.0</td>
<td>6.5\textsuperscript{C} ( \pm ) 4.5</td>
<td></td>
</tr>
<tr>
<td>\textit{F} \textit{3} ((\textit{Ant1C}/\textit{Ant1C}/\textit{hp-1}/\textit{hp-1})</td>
<td>5766.0\textsuperscript{A} ( \pm ) 1330.0</td>
<td>1162.0\textsuperscript{A} ( \pm ) 270.0</td>
<td>569.0\textsuperscript{A} ( \pm ) 139.0</td>
<td></td>
</tr>
<tr>
<td>\textit{F} \textit{3} ((\textit{Ant1F}/\textit{Ant1C}/\textit{hp-1}/\textit{hp-1})</td>
<td>1111.0\textsuperscript{B} ( \pm ) 122.0</td>
<td>289.4\textsuperscript{B} ( \pm ) 64.8</td>
<td>95.6\textsuperscript{B} ( \pm ) 13.5</td>
<td></td>
</tr>
<tr>
<td>\textit{F} \textit{3} ((\textit{Ant1C}/\textit{Ant1F}/\textit{hp-1}/\textit{hp-1})</td>
<td>0.0\textsuperscript{C} ( \pm ) 0.0</td>
<td>0.0\textsuperscript{C} ( \pm ) 0.0</td>
<td>0.0\textsuperscript{C} ( \pm ) 0.0</td>
<td></td>
</tr>
<tr>
<td>\textit{P}(\textit{F})</td>
<td>0.0005</td>
<td>0.0005</td>
<td>0.0006</td>
<td></td>
</tr>
</tbody>
</table>

SE, standard error. Values represent peak area per gram of fresh skin weight. Different superscript letters indicate statistically significant differences between means within each metabolite \((P < 0.05)\) according to the Tukey–Kramer HSD test (Kramer 1956). \textit{P}(\textit{F}) indicates the probability of \textit{F} value obtained by an analysis of variance.
The *Ant1* gene originating from *S. chilense* showed major differences in the coding sequence, leading to 8 amino acid substitutions at the predicted protein level (Figure 2). These substitutions were accompanied by a statistically significant difference in transcription of the *Ant1* gene in tomato peel taken from fruits harvested from LA1996 (*Aft*), as compared with the red-fruited Moneymaker counterparts (Table 3). Borovsky et al. (2004) also reported a complete association between the dominant *A* gene that accumulates anthocyanin pigments in immature pepper fruit and the gene homologous to the *Ant1* gene. In this case, *Ant1* was found to be transcribed at all stages of fruit development in the purple-fruited genotype, whereas in the green-fruited genotype, it was not transcribed at all. This transcriptional difference was attributed to variation in the promoter region because sequence comparison of *Ant1* between green- and purple-fruited genotypes revealed no differences in its coding region. Assuming that the *Ant1* gene is responsible for both *A* and *Aft* phenotypes, our results point to sequence differences and not transcriptional alteration as the cause of the *Aft* phenotype in the tomato. In this respect, it would be interesting to carry out a comparative transgenic study to compare the phenotypes resulting from overexpression of the *Ant1* genes originating from *S. chilense* and *S. lycopersicum*, driven by the same promoter.

Transcriptional profiling of key structural genes of the flavonoid biosynthetic pathway revealed that early and late genes of the pathway are induced in the *Aft* mutant in comparison to its red-fruit counterparts. These genes were also upregulated after *Ant1* activation (Mathews et al. 2003) as well as in the *A* genotype of pepper (Borovsky et al. 2004) and reinforce our hypothesis that *Ant1* is the gene controlling the anthocyanin and flavonol accumulation phenotype of the *Aft* genotype. In the current study, however, the *Ant1* gene did not display transcriptional upregulation in the *Aft* genotype, pointing again to changes in the amino acid sequence of the *Ant1* gene originating from *S. chilense* as being responsible for the *Aft* phenotype.

Interestingly, heterozygous *Aft/+* plants displayed higher transcriptional activation of *chs1, chs2*, and *dfr* than homozygous *Aft/Aft* plants (Table 3), whereas their average anthocyanin and flavonol concentrations were much lower and statistically insignificant from +/+ plants (Tables 1 and 2). It therefore seems that from a transcriptional point of view, the *Aft* allele has the potential to display a dominant or even an overdominant effect over the + allele. However, some posttranscriptional events may diminish this potential. One such event may be a competition at the protein level between *Aft* (possibly *Ant1c* originating from *S. chilense*) and + (possibly *Ant1e* originating from *S. lycopersicum*). Further, *Aft* from *S. chilense* and the + allele from *S. lycopersicum* may be controlled by divergent promoters, thus responding differently to environmental cues. Under certain environmental conditions, heterozygous plants may express more *Aft* than + transcripts, resulting in a dominant phenotype, whereas under different environmental conditions, + may be more highly expressed than *Aft* and result in a much weaker phenotype.

The codominant marker we developed and its strong association with *Aft* allowed us to map the *Aft* locus to the tomato chromosome 10. These results are in agreement with a previous study in which a DNA probe based on *An2* gene sequences, the *Petunia* homolog of *Ant1*, was used for mapping in tomatoes (De Jong et al. 2004). Noteworthy, our results contradict another study mentioned by Jones et al. (2003), in which 6 RFLP loci surveyed along chromosome 10 of LA1996 failed to uncover *S. chilense* introgressions.

The DNA marker developed in the course of this study enabled directed pyramiding of the *Aft* gene and the *hp-1* mutation, both enhancing fruit flavonoid content, into a single genetic background. Characterization of the flavonoid levels in fruits harvested from double homozygotes *Aftc/Afft hp-1/hp-1* plants revealed a synergistic effect of these 2 genes on the production of the anthocyanidins, delphinidin, petunidin, and malvidin, and the flavonols, quercetin and kaempferol, in the fruit. This effect was strongly manifested by approximately 5-, 19-, and 33-fold increase of petunidin, malvidin, and delphinidin, respectively, in the double mutants compared with the cumulative levels of their parental lines (Table 4). These results demonstrate the capacity of *hp* genotypes, known to share higher levels of several flavonoids (Bino et al. 2005, Levin et al. 2006), to further enhance levels of flavonoid metabolites when combined with another flavonoid-enhancing gene such as the *Aft*. This latter result is in agreement with a recent textbook report indicating that

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### Table 5. Average relative concentrations of major flavonols detected in ripe fruits of parental and F3 genotypes

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Quercetin (mean ± SE)</th>
<th>Kaempferol (mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1 (Ant1c/Ant1c +/+ )</td>
<td>6372.00 ± 666.5</td>
<td>1001.00 ± 32.0</td>
</tr>
<tr>
<td>P2 (Ant1e/Ant1e hp-1/hp-1)</td>
<td>21962.00 ± 3875.0</td>
<td>1371.00 ± 167.0</td>
</tr>
<tr>
<td>F1 (Ant1c/Ant1c hp-1/hp-1)</td>
<td>34016.00 ± 6778.0</td>
<td>2598.00 ± 519.7</td>
</tr>
<tr>
<td>F3 (Ant1c/Ant1e hp-1/hp-1)</td>
<td>26528.00 ± 2298.0</td>
<td>1928.00 ± 278.9</td>
</tr>
<tr>
<td>F3 (Ant1e/Ant1c hp-1/hp-1)</td>
<td>13413.00 ± 4415.0</td>
<td>791.23 ± 209.4</td>
</tr>
<tr>
<td>P(F)</td>
<td>0.0216</td>
<td>0.0187</td>
</tr>
</tbody>
</table>

SE, standard error. Values represent peak area per gram of fresh skin weight. Different superscript letters indicate statistically significant differences between means within each metabolite (*P* < 0.05) according to the Tukey–Kramer HSD test (Kramer 1956). *P(F)* indicates the probability of *F* value obtained by an analysis of variance.
several phenolic compounds with high antioxidant activity are new or increased in fruits of double mutants \textit{Aft/Aft hp-1w/hp-1w}, as compared with fruits of each of the single-locus mutants, and generalizes the value of genetically combining mutants at \textit{Aft} and \textit{hp} loci (van Tuinen et al. 2006).

In summary, results of this work strongly suggest that \textit{Ant1} is a likely candidate gene encoding the \textit{Aft} phenotype and confirm, on a quantitative basis, an earlier report showing that it can act synergistically with other \textit{hp} mutants to further increase fruit flavonoid levels (van Tuinen et al. 2006). Our results pave the way for directed introgression of the \textit{Aft} phenotype by marker-assisted breeding. The strong and-more-than-additive interaction obtained between \textit{hp} mutants, representing lesions in regulatory photomorphogenic genes (\textit{Det1} and \textit{Ddb1}), and a \textit{Myb} transcription factor point to modulation of regulatory networks as an additional resource for increasing fruit functionality. However, this final conclusion awaits final confirmation that \textit{Ant1} is the gene that causes the \textit{Aft} phenotype.

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