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EDITED BY
Prasenjit Saha,
Planet 13 Holdings, Inc., United States

REVIEWED BY
Ashutosh Mukherjee,
Vivekananda College, India
Thiya Mukherjee,
Donald Danforth Plant Science Center, United States

*CORRESPONDENCE
Silvia Gonzali
s.gonzali@santannapisa.it

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Transcriptional survey of the light-induced anthocyanin pathway in non-GM purple tomatoes

Silvia Gonzali*, Jacopo Menconi and Pierdomenico Perata

PlantLab, Institute of Plant Sciences, Scuola Superiore Sant'Anna, Pisa, Italy

Anthocyanins are polyphenolic compounds with antioxidant capacity, free radical scavenging power, and signaling activities in animal pathogenesis-associated pathways, thus playing an important role as nutraceuticals. Tomato fruits do not usually contain anthocyanins because their biosynthesis is switched off in these organs, but anthocyanin-enriched purple tomatoes have been produced in recent years. The varieties obtained by breeding express a functional copy of the R2R3-MYB transcription factor AN2-like, necessary to start the biosynthetic pathway, and do not produce a functional MYB-ATV repressor. The combination of these traits allows the accumulation of anthocyanins in tomatoes, strengthened under specific environmental factors such as high light intensity or low temperatures. Light starts anthocyanin synthesis and gradually extends its distribution on the fruit exocarp. The analyses carried out in the present study indicate that anthocyanin biosynthesis triggered by light is under HY5 control. However, the process is not active in mesocarp for the absence of the bHLH factor AN1, necessary to produce the MBW complex inducing the late enzymes of the biosynthetic pathway, as a consequence of insufficient expression of the R2R3-MYB gene *AN2-like*. This occurs since light cannot be perceived in the tissues underneath the skin because of the solar shield produced by the anthocyanins accumulated in the exocarp and for the activation of regulatory loops controlling HY5 levels. This is shown by the expression of genes involved in the production of photoreceptors and in the light signaling chain operating upstream of the anthocyanin pathway and responsible for its activation.

KEYWORDS

***Solanum lycopersicum*, purple tomato, anthocyanins, MBW, DEETIOLATED 1 (DET1), ELONGATED HYPOCOTYL 5 (HY5), photoreceptors, light signaling**

Introduction

In recent years, increased or *de novo* anthocyanin accumulation in fruits and vegetables have been pursued to enrich their nutraceutical value (Martin et al., 2011; Mattoo et al., 2022). Tomato (*Solanum lycopersicum* L.) has been improved in some nutritional traits (Raiola et al., 2014), and the establishment of the anthocyanin biosynthetic pathway in fruits is one of them (Butelli et al., 2008; Gonzali et al., 2009).

Anthocyanins are soluble polyphenols belonging to the class of flavonoids, representing the glycosylated forms of the corresponding anthocyanidins. They are characterized by different colors, from orange/red to purple/blue, and are involved in multiple functions, from the pigmentation of flowers and fruits to the protection of plants from biotic and abiotic stresses (Landi et al., 2015; Alappat and Alappat, 2020). In tomato fruits, their presence confers a dark purple color due to the prevalence of anthocyanidins belonging to the delphinidin class (Tohge et al., 2015; Blando et al., 2019). In leaves, the major role of anthocyanins is carried out in epidermal and subepidermal cell layers, where they act as a screen to filter solar radiation harmful for the photosynthetic apparatus, particularly under conditions of high irradiance and low temperatures, that can produce free radical species and photoinhibition (Gould, 2004). In fruits, their presence is often a marker of ripening to attract seed dispersers and is controlled by developmental programs (Jaakola, 2013). However, the synthesis of anthocyanins in fruits may also be induced by light and other environmental factors to better modulate their quantity and/or quality (Jaakola, 2013; Zoratti et al., 2014). The biosynthetic process starts from the aromatic amino acid phenylalanine and proceeds with a concerted series of enzymatic reactions whose early steps are in common with other flavonoids (Winkel-Shirley, 2001). The "structural" genes encoding the enzymes of the pathway are conserved in plants (Sunil and Shetty, 2022); more variable is their regulation, being the pathway controlled by distinct factors in different organs or tissues.

Light may play a primary role in the induction of anthocyanin synthesis, and its quality is particularly important in determining the quantity of anthocyanins produced and their nature (Ma et al., 2021). Among the radiations of the solar spectrum, UV and blue light mostly affect these processes, but red light can induce anthocyanin synthesis as well (Costa Galvão and Fankhauser, 2015; Podolec and Ulm, 2018; Rai et al., 2021). For this reason, multiple photoreceptors may play important roles as mediators between light and pigment production (Zoratti et al., 2014). Higher plants perceive surrounding solar radiations through different photoreceptors, including UV-B Resistance 8 (UVR8) for UV-B/UV-A light (Rai et al., 2021), cryptochromes for UV-A/blue light, and phytochromes for red/far-red light (Costa Galvão and Fankhauser, 2015; Podolec and Ulm, 2018). Downstream, a complex signaling mechanism takes place, with the basic leucine zipper transcription factor (TF) ELONGATED HYPOCOTYL 5 (HY5) playing the role of the master switching molecule (Gangappa and Botto, 2016).

HY5 constitutes the center of a transcriptional network hub regulating the expression of hundreds of different light-responsive genes in plants, including anthocyanin regulatory and biosynthetic genes (Lee et al., 2007; Shin et al., 2013). With HY5 being destabilized in dark conditions through the proteasome machinery activated by the CONSTITUTIVE PHOTOMORPHOGENESIS 1 (COP1)/SUPPRESSOR OF PHYTOCHROME A-105 (SPA) ubiquitin ligase complex (Park et al., 2017; Bian et al., 2022; Shin et al., 2007), anthocyanin synthesis can be switched off in absence of light (Albert et al., 2009; Kim et al., 2017). Also, shaded conditions and high temperatures may destabilize HY5 through the COP1/SPA complex (Sheerin and Hiltbrunner, 2017; Nieto et al., 2022), leading to reduction or suppression of anthocyanin production (Lin-Wang et al., 2011). *Arabidopsis cop1* mutant seedlings can accumulate exaggerated quantities of anthocyanins in cotyledons, showing the so-called "FUSCA" phenotype, due to the stabilization of HY5 (Castle' and Meinke, 1994; Han et al., 2020). COP10, DEETIOLATED 1 (DET1), and UV-damaged DNA-binding protein 1a (DDB1a) constitute the CDD complex, which physically interacts with COP1/SPA and the COP9 signalosome, allowing their activity (Yanagawa et al., 2004; Lau and Deng, 2012; Cañibano et al., 2021). DET1 is a chromatin-associated protein acting as a transcriptional repressor in dark conditions; its mutation, similarly to *COP1* mutation, results in a stabilization of HY5 either in light or dark, with the consequent ectopic activation of HY5 targets (Cañibano et al., 2021). In tomato fruit, HY5 regulates ripening at both transcriptional and translational levels because many genes involved in carotenoid and flavonoid biosynthesis and in ethylene signaling are HY5 targets (Wang et al., 2021). Consequently, due to stabilization of HY5, *DET1* mutants, known as *high pigment 2* (*hp2*) in tomato, show enhanced phenylpropanoids, flavonoids, and carotenoids in their fruits (Mustilli et al., 1999).

The last decades have seen a significant advance in understanding the regulatory mechanisms underlying the anthocyanin production, in particular the role of the R2R3-MYB TFs which activate the *early biosynthetic genes* (*EBGs*) and of the "MBW" multiprotein complexes which mainly act on the *late biosynthetic genes* (*LBGs*) (Albert et al., 2014; Xu et al., 2015; Lloyd et al., 2017). The MBW complex is composed of R2R3-MYB, bHLH, and WDR factors, with the R2R3-MYB mainly conferring transcriptional specificity on the genomic targets via interaction with the MYB recognition elements (MREs) in their promoters (Albert et al., 2014; Xu et al., 2015). In tomato, the main R2R3-MYB protein activating the *EBGs* is MYB12, whereas the MBW complexes, which act in a hierarchical way (Montefiori et al., 2015), are composed by the R2R3-MYB AN2 or AN2-like (acting in vegetative tissues and fruits, respectively), the bHLH factors JAF13 and AN1, and the WDR protein AN11 (Kiferle et al., 2015; Qiu et al., 2016; Gao et al., 2018; Colanero et al., 2020; Sun et al., 2020). Repressor R2R3-MYB and R3-MYB factors can act independently or via interaction with the MBW complex to destabilize its transcriptional activations and thus inhibit or decrease the anthocyanin production (LaFountain and Yuan, 2021). Among the tomato R2R3-MYB repressors, THM27 (also known as MYB32), MYB76, and MYB72 have been recently found to be involved in inhibition of both *EBGs* and *LBGs*, even if with different specificities (Menconi et al., 2023; Suprun et al., 2023; Wu et al., 2020). MYB-ATV is a tomato CPC-type R3-MYB repressor which can sequester the bHLH proteins from the MBW complex and is transcriptionally induced by the same complex, triggering in this way a feedback repression mechanism (Colanero et al., 2018). Other

tomato R3-MYB repressors are MYB-ATV-like and TRIPTYCHON (TRY) (Nukumizu et al., 2013; Cao et al., 2017). The regulation of the anthocyanin pathway is further affected by other proteins, acting under environmental or developmental control (e.g., WRKYs, SPLs, DELLAs, JAZs), which can bind the MBW complexes with positive or negative effects on their final activity (Xu et al., 2015; Lloyd et al., 2017). In anthocyanin-enriched tomato fruits, several other TFs have also been identified in recent years as positively correlated under light with anthocyanin pigmentation, some of them acting independently of HY5 (Qiu et al., 2019), and others under hormonal control (You et al., 2024).

The introgression in *S. lycopersicum* of the *Anthocyanin fruit* (*Aft*) or the *Aubergine* (*Abg*) alleles from *Solanum chilense* (Georgiev, 1972) or *Solanum lycopersicoides* (Rick et al., 1994), respectively, allows the activation of the synthesis of spotted anthocyanin pigmentation in the fruit exocarp (Mes et al., 2008; Menconi et al., 2024). This revealed the criticality of the fruit-specific R2R3-MYB encoding gene *AN2-like*, of which both *Aft* and *Abg* are functional alleles, whereas the *S. lycopersicum* sequence bears a splicing mutation which produces a non-functional TF (Colanero et al., 2020; Sun et al., 2020; Menconi et al., 2023, 2024). The locus *atroviolacea* (*atv*), introgressed from *Solanum cheesmaniae* (Rick, 1964), revealed the existence of MYB-ATV, which, when mutated, leads to derepressed anthocyanin accumulation either in fruits or in vegetative tissues (Cao et al., 2017; Colanero et al., 2018). The concomitant presence of *Aft* or *Abg* and *atv* leads under light to biosynthesis and enhanced accumulation of anthocyanins in the epidermal and subepidermal cell tissues of pericarp from the early stages of fruit ripening (Mes et al., 2008; Povero et al., 2011; Sun et al., 2020; Menconi et al., 2024). This generally does not occur in mesocarp, which remains green for the presence of chlorophylls in early stages and becomes red in ripe fruits when carotenoids accumulate (Mes et al., 2008; Povero et al., 2011; Menconi et al., 2024). However, the expression of a functional copy of *AN2-like* under the *E8* fruit promoter led to tomatoes with strong anthocyanin pigmentation in all the fruit (Sun et al., 2020). The same occurred through overexpression of R2R3-MYB paralogs of AN2-like, such as AN2 (Kiferle et al., 2015; Jian et al., 2019). This suggests that anthocyanin biosynthesis is a cell autonomous process in tomato fruit and requires local presence of a suitable R2R3-MYB factor able to interact with JAF13 and AN11, respectively, the bHLH and WDR partners of the first MBW complex (MBW1), which are expressed independently of anthocyanins (Povero et al., 2011; Kiferle et al., 2015; Gao et al., 2018), to activate transcription of the bHLH gene *AN1* (Montefiori et al., 2015), whose protein replaces JAF13 producing the second MBW complex (MBW2), which finally activates the expression of the *LBGs*.

The objective of the present study is to describe in detail the transcriptional activities, from the light stimulus downward, which in purple tomato fruit allow and not allow, respectively, anthocyanin synthesis in exocarp and internal tissues under light, by analyzing well-known regulators of the pathway as well as novel TFs recently hypothesized to be involved in the process, to understand which are the most critical steps and how such drawbacks may be overcome.

Materials and methods

Plant material and growth conditions

The tomato genotypes *Aft/Aft* × *atv/atv (Aft/atv)* and *Aft/Aft* × *atv/atv* × *hp2/hp2 (Aft/atv/hp2)* in the MicroTom background were used. The seeds of the line *Aft/atv/hp2*, containing the allele *dark green* of the *DET1* gene (Levin et al., 2003), were donated by Prof. Peres (Sestari et al., 2014). The seeds of *Aft/atv* were obtained by backcrossing *Aft/atv/hp2* with the cv. MicroTom and selecting for the *Aft/atv* genotype in the segregating F2 and F3 generations. Seeds were germinated in rock-wool plugs (Grodan) soaked in a nutritive solution (Kiferle et al., 2015). 2-week-old seedlings were transplanted in pots containing a 70:30 soil (HAWITA-Flor)/expanded clay mixture and placed in a growth chamber with 23°/20°C of day/night temperature, 12-h photoperiod, 150 µmol photons m^{-2} s^{-1}, and 40% relative humidity. For both anthocyanin quantification and qPCR analysis, fruits were sampled at mature green or mature red stages with exocarp (containing also the cuticle layer) removed from the stem end and the inner parts, constituted by mesocarp and endocarp, collected without placenta and seeds. Biological replicates corresponded to fruits collected from independent plants at the same developmental stage, similar position within the plant and similar light exposition, to reduce the pigmentation variability as much as possible. The material was frozen in liquid nitrogen and stored at −80°C until use.

Anthocyanin quantification

Anthocyanin extraction was performed starting from 50 mg of fruit material (exocarp or mesocarp + endocarp). Fruit samples were ground in 300 µl of HCl 1% (v/v) in methanol and incubated with gentle agitation overnight at 4°C. Extracts were recovered, and 200 µl of distilled water was added. 1 volume of chloroform was then added to remove chlorophylls through mixing and centrifugation (1 min at 14,000 × g). The aqueous phase containing anthocyanins was recovered, 600 µl of HCl 1% (v/v) in methanol was added, and absorption was determined spectrophotometrically. Relative anthocyanin concentrations were calculated as a difference between the absorbance read at 530 nm and 657 nm (Neff and Chory, 1998) and finally expressed as microgram petunidin-3-(p-coumaroyl rutinoside)-5-glucoside $gram^{-1}$ fresh weight. Mean values were obtained from three independent replicates consisting of fruit material collected from different plants (one fruit per plant).

RNA isolation, cDNA synthesis, and qPCR analysis

Total RNA was extracted from fruit exocarp or mesocarp + endocarp with the "Spectrum™ Plant Total RNA Kit" (Merck). The quality of RNA was assessed through electrophoresis on 1% agarose gels, and the quantity was measured through a µDrop™ plate on a Multiskan microplate reader (Thermo Scientific). One microgram of RNA was subjected to DNase treatment and then reverse transcribed into cDNA using the "Maxima First Strand cDNA Synthesis Kit for RT-qPCR, with dsDNase" (Thermo Fisher Scientific) and subsequently diluted with nuclease-free water (Merck) to a concentration of 5 ng/µl. Quantitative RT-PCR (qPCR) was performed with an ABI Prism 7300 Sequence Detection System (Thermo Fisher Scientific). qPCR reactions were carried out using the "PowerUp™ SYBR® Green Master Mix" (Thermo Fisher Scientific), 15 ng of cDNA template, and 300 nM forward and reverse primers (listed in Supplementary Table S1), in a final reaction volume of 10 µl. No-template control reactions were performed for each pair of primers. Amplicon dissociation curves were recorded to confirm the gene specific amplification by a single dominant peak. *Elongation Factor 1-alpha* (*EF1A*) and *Abscisic Acid Stress Ripening 1* (*ASR1*) (Bovy et al., 2002) were used as reference genes. The relative quantitation of each individual gene expression was performed using the geometric averaging method (geNorm) (Vandesompele et al., 2002). The relative expression level was calculated as ratio between quantity of target gene and quantity of the geometric averaging of the reference genes. Values are means of four biological replicates with two technical replicates for each gene.

Promoter analysis

The analysis of the cis-acting regulatory elements contained in the 3-kb genomic region upstream of the ATG first codon of the CDS of the anthocyanin MBW regulatory genes was carried out using the database of PlantCAre (https://bioinformatics.psb.ugent.be/webtools/plantcare/html/) (Rombauts et al., 1999; Lescot et al., 2002) with the Sol Genomics (https://solgenomics.org) id of the genes of interest.

Gene expression analysis in wild-type fruit

The expression analysis of the genes of interest in the fruit tissues of *S. lycopersicum* was checked by using the online tool "Tomato Expression Atlas" (https://tea.solgenomics.net/) (Pattison et al., 2015; Fernandez-Pozo et al., 2017; Shinozaki et al., 2018).

Statistics

Statistical analyses were performed with GraphPad Prism 6.01 (www.graphpad.com/scientific-software/prism/). Unpaired t-test or one-way ANOVA with Tukey's HSD *post-hoc* test was used to compare the means of two or more than two group samples, respectively.

Results

The *hp2* mutation increased the anthocyanin production in the exocarp of purple tomato fruits

To study the anthocyanin biosynthesis induced by light in anthocyanin-enriched tomato fruits, the genotypes *Aft/atv* and *Aft/atv/hp2* were used (Sestari et al., 2014). Tomato fruit pericarp was divided into two different samples: exocarp, containing the cuticle layer, the epidermal cell layer, and the underlying thin collenchymatous tissue, and the internal parts, constituted by the intermediate parenchymatous mesocarp and by the endocarp, consisting of a cell layer covering the locular cavities (Pesaresi et al., 2014). For sake of conciseness, however, from here on, the internal layers of the pericarp will be indicated with the single word "mesocarp". At the mature green (MG) stage, both *Aft/atv* and *Aft/atv/hp2* fruits showed accumulation of anthocyanins in the exocarp, with a more intense and evenly distributed pigmentation on *Aft/atv/hp2* fruits (Figures 1A, C, E). Instead, in both lines, anthocyanins were not present in mesocarp, as well as in the locular cavities, placenta, and developing seeds (Figures 1B, D, E).

The accumulation of anthocyanins was the likely consequence of activation of the biosynthetic pathway (Supplementary Figure S1): this occurred in early stages of fruit development (Supplementary Figure S2), and in MG fruits anthocyanins were already present at high levels (Figure 1E). The structural genes encoding enzymes involved in early and late reactions (Supplementary Table S2) resulted indeed expressed at MG (Figure 2A). In general, the expressions of the structural genes in *Aft/atv/hp2* exocarp were higher than in *Aft/atv* exocarp (Figure 2A), even if the differences were not always statistically significant. In mesocarp, on the contrary, in both lines all the biosynthetic genes were not expressed (Figure 2A), thus explaining the lack of anthocyanins in this part of the fruit.

The activator regulatory genes (Supplementary Table S2), either *MYB12* or the genes encoding the MBW factors, were transcribed in the exocarps of both lines and much less or even not in the relative mesocarps, with the only exception of *JAF13* which showed similar expressions in the two parts of the pericarp (Figure 2B). Excluding *MYB12* and *JAF13*, which were expressed at similar levels in the exocarps of the two lines, the other MBW regulatory genes were expressed in *Aft/atv/hp2* exocarp more than in *Aft/atv* (Figure 2B).

The genes encoding the repressor factors MYB-ATV, MYB-ATV-like, THM27, and MYB76 (Supplementary Table S2) were all more expressed in exocarps than in mesocarps and in *Aft/atv/hp2* exocarp more than in *Aft/atv* exocarp. A similar trend was shown by *WRKY44* and *GL2*. On the contrary, *ERF.G3-like* was not expressed in MG fruits (Figure 2B).

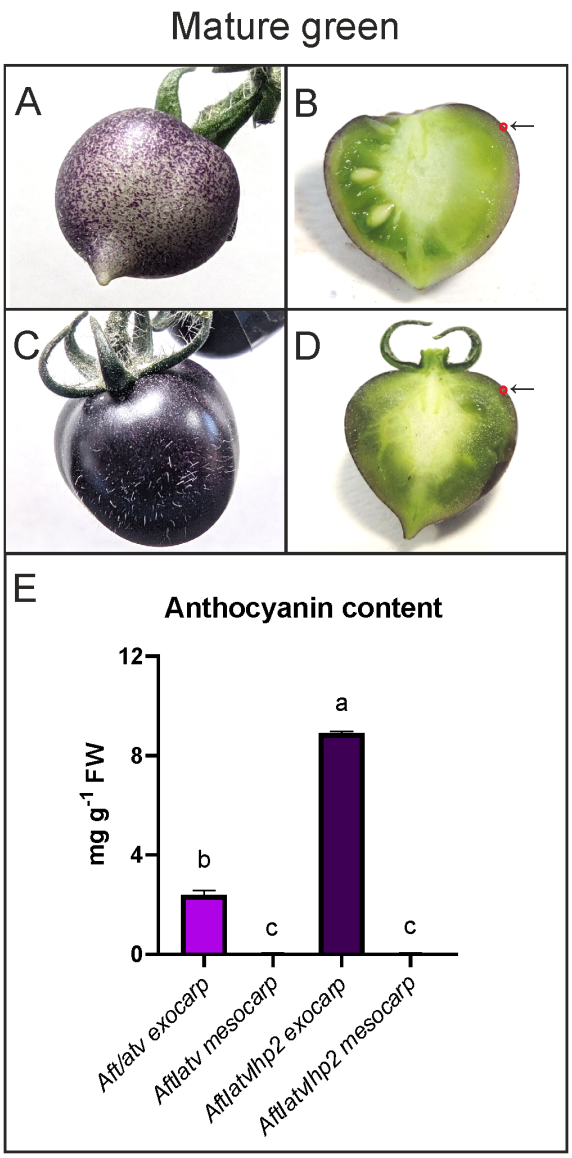

FIGURE 1
Phenotypes of anthocyanin-enriched tomato fruits at the mature green stage. *Aft/atv* **(A, B)** and *Aft/atv/hp2* fruits **(C, D)**, entire and in cross sections, respectively, were shown. In B and D the arrows indicate the site of accumulation of the anthocyanins in the exocarp, marked with a red circle. Anthocyanins measured in exocarp and mesocarp of *Aft/atv* and *Aft/atv/hp2* fruits, expressed as petunidin-3-(p-coumaroyl rutinoside)-5-glucoside per gram exocarp fresh weight (FW) **(E)**. Data are means (± SEM) of three biological replicates. One-way ANOVA with Tukey's HSD *post-hoc* test was performed. Different letters indicate significant differences at $p \leq 0.05$.

The light photoreceptors and the light signaling genes were differentially expressed in the fruits of the two genotypes

Genes encoding photoreceptors or their apoproteins were analyzed to highlight possible variations in the expression patterns between exocarp and mesocarp. All the genes under study, including *UVR8*, *cryptochrome 1a* (*CRY1a*), *cryptochrome 1b* (*CRY1b*), *cryptochrome 2* (*CRY2*), *cryptochrome DASH* (*CRY-DASH*), *phytochrome A* (*PhyA*), *phytochrome B1* (*PhyB1*), and *phytochrome B2* (*PhyB2*) (Supplementary Table S2), resulted to be expressed in both *Aft/atv* and *Aft/atv/hp2* fruits at MG, in either exocarps or mesocarps, with transcription levels in mesocarp generally lower than in exocarp (Figure 2C).

A common element downstream of the photoreceptors is HY5, but also other signaling factors, such as the B-BOX containing proteins (BBXs), may play important roles in relation to anthocyanin synthesis (Lee et al., 2007; Binkert et al., 2014; Xu, 2020; Wang et al., 2021; Menconi, 2024). The expressions of different genes known to code for key factors of the light signaling pathway (Supplementary Table S2) were analyzed. At MG, the expressions of *HY5* in exocarps were similar in *Aft/atv/hp2* and *Aft/atv* fruits, and potential HY5 target genes (Binkert et al., 2014; Burko et al., 2020), such as *COP1 homolog*, *COP1-like isoform X1*, and *REPRESSOR OF UV-B PHOTOMORPHOGENESIS* (*RUP*), also showed similar transcription levels in exocarp in the two genotypes (Figure 2D). While in *Aft/atv* fruits the transcription of all these genes was higher in exocarp than in mesocarp, in *Aft/atv/hp2* fruits this occurred for *HY5* and *COP1-like isoform X1*, whereas *COP1 homolog* and *RUP* showed the same expressions in exocarp and mesocarp (Figure 2D). Relative to the BBX encoding genes, they showed overall similar expression levels in the two lines with no big differences between exocarp and mesocarp, except for *BBX21* which appeared downregulated in *Aft/atv/hp2* exocarp compared with the other samples (Figure 2D).

Anthocyanin levels and gene expressions in ripe fruits

At the mature red (MR) stage, the fruits of the two lines showed uniform anthocyanin pigmentation of the exocarp (Figures 3A, C), whereas the internal parts appeared homogeneously red for the presence of lycopene and absence of anthocyanins (Figures 3B, D, E). The *Aft/atv/hp2* fruits still contained more anthocyanins in the exocarp than the other genotype (Figure 3E), but the quantity of pigments accumulated in the exocarp from MG to MR resulted higher in the *Aft/atv* line (Supplementary Figure S3).

At the transcriptional level, comparing MR with MG, a clear attenuation was visible in the expression of most of the genes analyzed. In the exocarp of the *Aft/atv* fruits, the reduction of the biosynthetic pathway activity was particularly strong (Figure 4A), and evident was also the decrease in the expressions of the regulatory genes *MYB12*, *AN2-like*, and *JAF13* among the activators, and *MYB-ATV*, *MYB-ATV-like*, and *THM27* among the repressors (Figure 4B). In *Aft/atv/hp2* exocarp, *MYB12* and *JAF13* expressions decreased at MR compared with MG, as well as *THM27*, but the other regulatory MBW genes continued to be expressed at high levels (Figure 4B). Interestingly, the regulatory gene *ERF.G3-like*, whose expression in MG fruits was not detected, resulted highly transcribed in MR fruits, in both mesocarps and exocarps, particularly in the *Aft/atv/hp2* fruit (Figure 4B).

The transcription of the genes encoding photoreceptor proteins appeared overall reduced in MR fruits compared with MG, still

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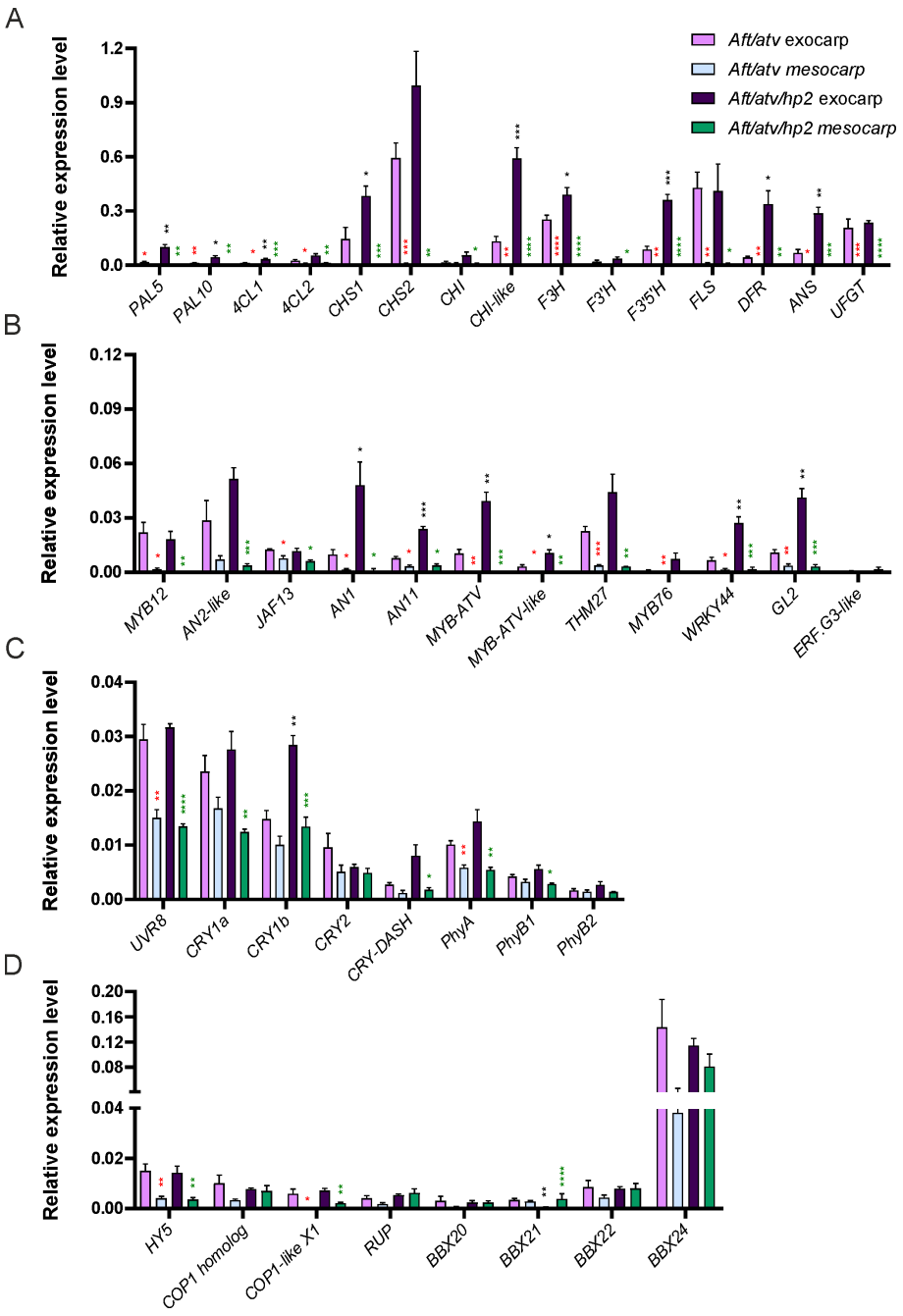

FIGURE 2
Relative expression levels of structural genes **(A)**, regulatory genes **(B)**, apoproteins of photoreceptors encoding genes **(C)** and light signaling genes **(D)** affecting or involved in the anthocyanin biosynthetic pathway, measured by qPCR in *Aft/atv* and *Aft/atv/hp2* tomato fruit exocarp and fruit mesocarp at the mature green stage. Data are means of four biological replicates ± SEM. For each gene expression analysis, t-test was performed between exocarp and mesocarp of *Aft/atv* fruits (red asterisks), exocarp and mesocarp of *Aft/atv/hp2* fruits (green asterisks), and *Aft/atv* exocarp and *Aft/atv/hp2* exocarp (black asterisks). Statistical significance is reported in function of the number of asterisks: *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001.

maintaining the same relative differences between the two genotypes and between exocarp and mesocarp previous observed (Figure 4C). The reduction of mRNA levels compared with MG was particularly evident for the gene *UVR8*, *CRY1a*, *CRY1b*, and *PhyB1*.

Finally, as for the light signaling genes, most of them resulted to be still expressed in MR fruits but with some differences compared with MG. First of all, *HY5* was less transcribed in the exocarps of both lines than at MG and showed a higher expression in *Aft/atv/hp2* fruits than in *Aft/atv* (Figure 4D). The mRNA levels of *COP1 homolog* slightly increased in the fruit mesocarp in both lines, whereas *COP1-like isoform X1* (whose expression was already low in the mesocarps of MG fruits) resulted to be not expressed also in

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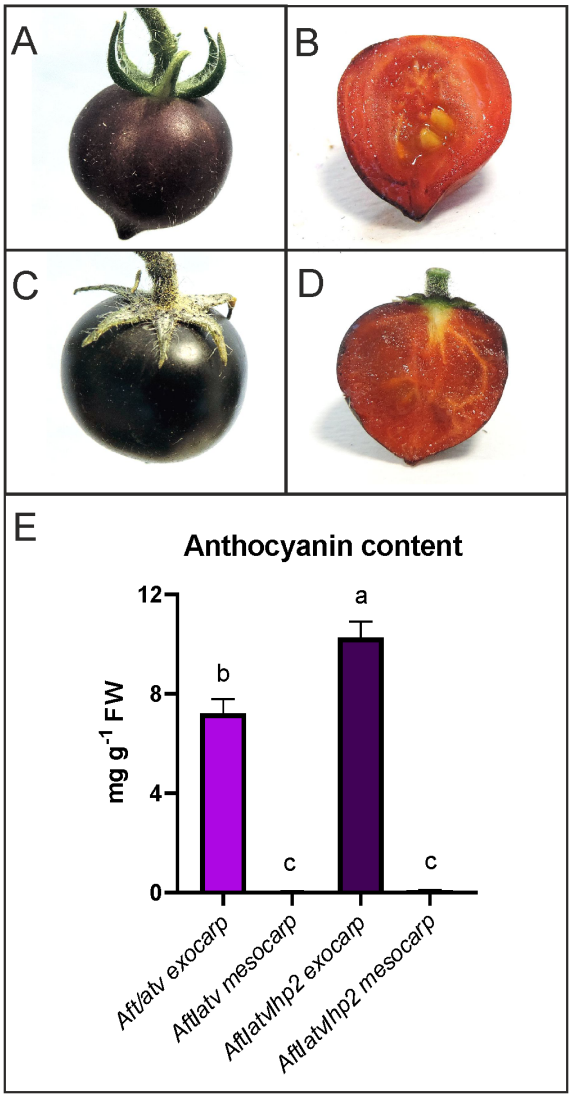

FIGURE 3
Phenotypes of anthocyanin-enriched tomato fruits at the mature red stage. *Aft/atv* **(A, B)** and *Aft/atv/hp2* fruits **(C, D)**, entire and in cross sections, respectively, were shown. Anthocyanins measured in exocarp and mesocarp of *Aft/atv* and *Aft/atv/hp2* fruits, expressed as petunidin-3-(p-coumaroyl rutinoside)-5-glucoside per gram exocarp fresh weight (FW) **(E)**. Data are means (± SEM) of three biological replicates. One-way ANOVA with Tukey's HSD *post-hoc* test was performed. Different letters indicate significant differences at $p \leq 0.05$.

the exocarp of the *Aft/atv* MR fruits, and very low levels of its mRNA were detected only in the exocarp of the *Aft/atv/hp2* fruits (Figure 4D). However, the strongest variations in gene expression between the two developmental stages interested *RUP*, whose expression strongly increased in *Aft/atv/hp2* fruits, particularly in the exocarp, and *BBX21*, whose mRNA dropped at barely detectable levels in both genotypes. On the contrary, *BBX20* and *BBX22* expressions increased in all the fruit tissues of both lines, whereas *BBX24* remained well expressed in all the samples, but its levels decreased in the *Aft/atv* exocarp compared with MG (Figure 4D).

Trans-activation of the anthocyanin regulatory genes by light and other factors

Being the regulatory genes encoding the components of the MBW complexes transcriptionally activated by TFs acting upstream, an analysis on their promoter sequences was performed and different classes of cis-acting responsive elements were identified (Figure 5). In the light-mediated activation of the anthocyanin pathway, the most important classes of cis-acting sequences are the light-responsive elements (LREs), which were indeed identified in the genomic regions upstream of the CDS of all the regulatory genes. Among them, specific HY5 binding sites, belonging to both G-Box and other ACE-Box classes, were found. Several MYB responsive elements were also present and MREs specifically involved in light responsiveness were identified.

Other environmental factors may induce anthocyanin biosynthesis besides light, and cold temperatures are known to be able to stimulate anthocyanin production in tomato leaves (Kiferle et al., 2015). Low temperature-responsive (LTR) elements were in fact present in the promoters of *AN11* and *AN1* genes, but not in those of *AN2-like* and *JAF13* (Figure 5).

Finally, several hormonal responsive-elements were found, and ethylene-responsive elements (EREs), which may link anthocyanin biosynthesis with ethylene production occurring during fruit ripening, were identified in the promoters of *AN2-like*, *AN11*, and *AN1* (Figure 5).

Discussion

The anthocyanin biosynthetic pathway is HY5-dependent and fine-tuned by feedback repression mechanisms in tomato fruits

When the *Aft* and *atv* alleles are present in the tomato genome, under adequate light intensities the flavonoid biosynthetic pathway produces anthocyanins in the fruit exocarp. In the experimental setup used in this work, light activated the anthocyanin synthesis in immature fruits (Supplementary Figure S2), and the quantity of pigments increased in exocarp till MR (Supplementary Figure S3). As expected, both *EBGs* and *LBGs* were expressed in the exocarp, likely induced, respectively, by MYB12 (Adato et al., 2009; Ballester et al., 2010) and by the MBW complexes (Montefiori et al., 2015), whose genes were all expressed at MG (Figures 2A, B). From MG on, some activators of the pathway tended to reduce their expression in *Aft/atv* exocarp, and this was followed by a general reduction in the expression of the biosynthetic genes (Figures 4A, B). In *Aft/atv* fruit mesocarp, anthocyanins were not produced at both MG and MR for the lack of expression of the structural genes, accompanied by reduced or null transcription of the regulatory factors (Figures 2A, B, 4A, B). The mutation *hp2* increased the anthocyanin accumulation in *Aft/atv* fruit exocarp, as a consequence of higher activation of most regulatory and structural genes at both MG and MR but, remarkably, did not

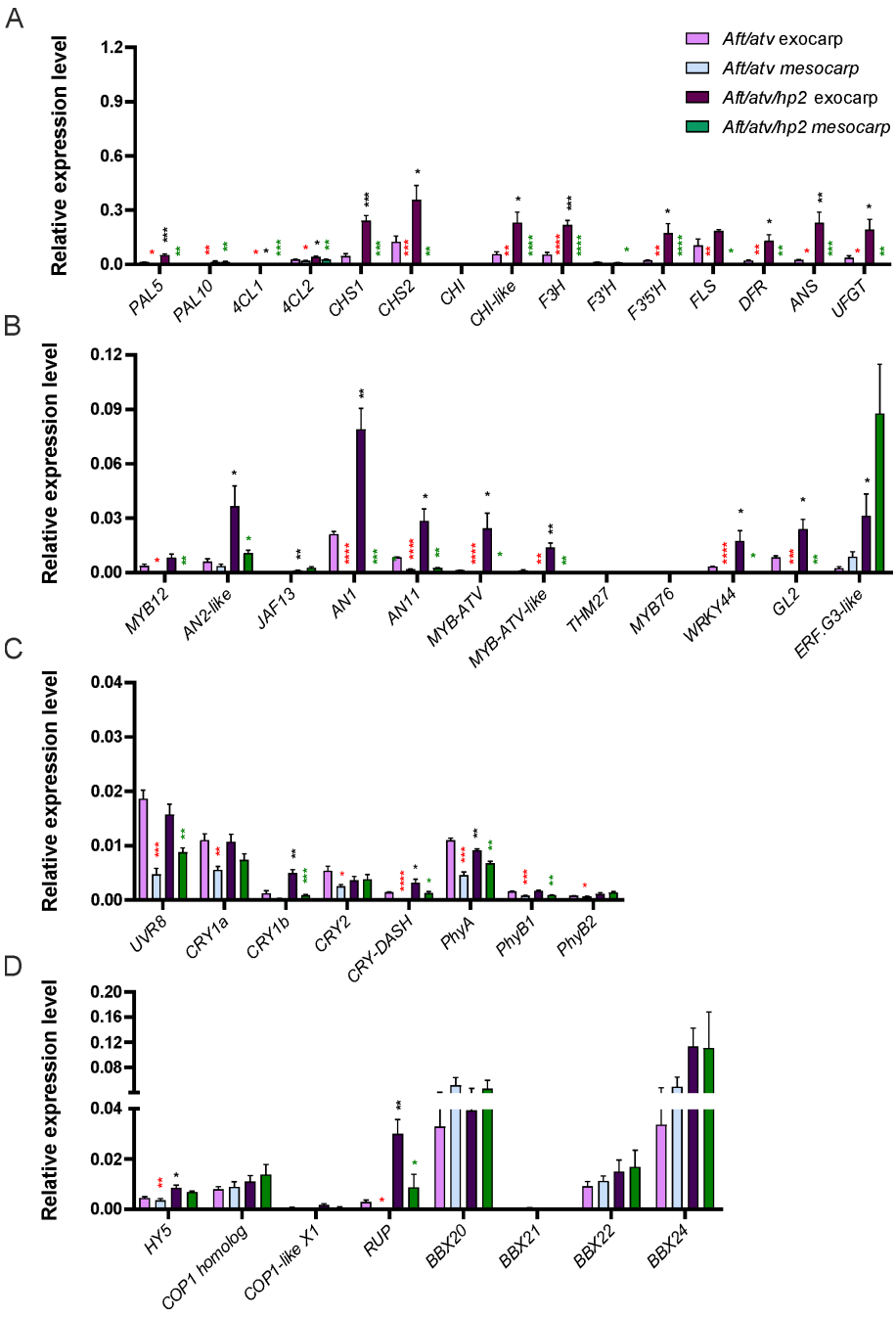

FIGURE 4
Relative expression levels of structural genes **(A)**, regulatory genes **(B)**, apoproteins of photoreceptors encoding genes **(C)**, and light signaling genes **(D)** affecting or involved in the anthocyanin biosynthetic pathway, measured by qPCR in *Aft/atv* and *Aft/atv/hp2* tomato fruit exocarp and fruit mesocarp at the mature red stage. Data are means of four biological replicates ± SEM. For each gene expression analysis, t-test was performed between exocarp and mesocarp of *Aft/atv* fruits (red asterisks), exocarp and mesocarp of *Aft/atv/hp2* fruits (green asterisks), and *Aft/atv* exocarp and *Aft/atv/hp2* exocarp (black asterisks). Statistical significance is reported in function of the number of asterisks: *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001.

affect anthocyanin synthesis in the fruit mesocarp (Figures 2A, B, 4A, B). Interestingly, not only were the genes analyzed expressed in *Aft/atv/hp2* exocarp generally more than in *Aft/atv* exocarp (Supplementary Figure S4, columns A and D) but also the ratios of gene expression between mesocarp and exocarp resulted lower in *Aft/atv/hp2* fruits than in *Aft/atv* fruits for many of them (Supplementary Figure S4, columns B, C, E, F).

The *DET1/hp2* mutation, impairing the COP1/SPA ubiquitin ligase activity, stabilizes HY5 and induces its targets, thus conferring hypersensitivity to light (Mustilli et al., 1999; Lau and Deng, 2012; Cañibano et al., 2021). The stabilization of HY5 in the exocarp of *Aft/atv/hp2* fruits was proved by the increased expression at MR of some of its known targets, such as *COP1 homolog* and *COP1-like isoform X1* (Burko et al., 2020; Menconi, 2024), *RUP* (Binkert et al.,

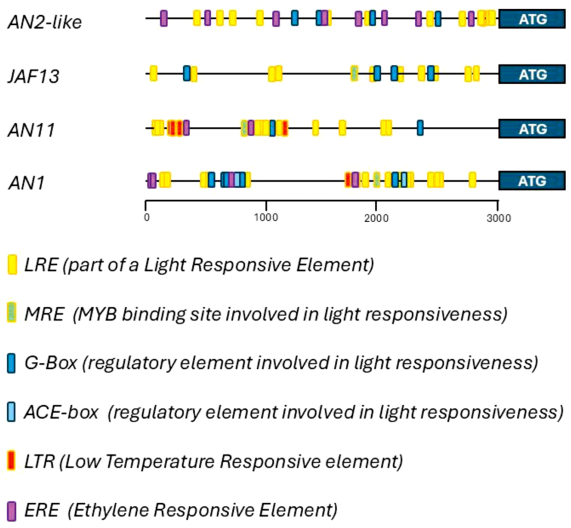

FIGURE 5
Cis-acting regulatory elements in the promoters of the genes involved in the production of the tomato MBW complexes. Light-responsive elements (LRE) (yellow), MYB regulatory elements (MRE) involved in light responsiveness (green), G-Box regulatory elements (blue), other ACE-box cis-acting regulatory elements involved in light responsiveness (light blue), low-temperature responsive (LTR) elements (red), and ethylene-responsive elements (EREs) (purple) identified in the 3-kb regions upstream of the ATG first codon in the genomic sequences of the regulatory genes *AN2-like*, *JAF13*, *AN11*, and *AN1*. The analysis of the regulatory elements was carried out using the database of "Plant CARE" web site.

2014; Zhang et al., 2021), and the same *HY5* gene (Figure 4D), which is prone of autoactivation (Binkert et al., 2014). Likewise, the increased transcription of almost all the genes encoding the regulatory factors and the biosynthetic enzymes in *Aft/atv/hp2* exocarp compared with *Aft/atv* exocarp indicated that the light-dependent regulation of the anthocyanin pathway in purple tomato fruit is mediated by HY5.

The transcription of the MBW genes *AN2-like* and *AN1* was specifically induced in *Aft/atv* fruits by light, being null their expressions in wild-type fruits (Supplementary Figure S5). Conversely, the other MBW genes *JAF13* and *AN11* are expressed also in wild-type fruits independently from the presence of anthocyanins (Supplementary Figure S6). The transcriptions of *AN2-like*, *AN1*, and *AN11* resulted upregulated in *Aft/atv/hp2* fruits and their promoters showed the presence of G-box and ACE-box sequences, putative cis-acting HY5 binding sites (Shin et al., 2013; Binkert et al., 2014; Zoratti et al., 2014) (Figure 5): thus, they might be directly targeted by HY5.

The induction by light of *AN2-like* switched on the hierarchical regulatory chain which produced the MBW1 complex through interaction of AN2-like with JAF13 and AN11, constitutively present (Supplementary Figure S6). *AN1* contains in its promoter both LREs and MREs involved in light responsiveness (Figure 5). Similarly to the light-mediated induction of other genes of the flavonoid pathway, which require MREs as part of the light-responsive units (Hartmann et al., 2005), it is possible that the activation of *AN1* requires the concerted action of the MBW1 complex, binding its promoter through AN2-like, with HY5. The same mechanism might allow transcription of the structural genes, being activated, respectively, by MYB12 and by the MBW2 complex, but also directly bound in their promoters by HY5 (Wang et al., 2021): as a confirmation, both *EBGs* and *LBGs* resulted to be more expressed in *Aft/atv/hp2* than in *Aft/atv* exocarp (Figure 2A). Furthermore, feedback repression mechanisms (LaFountain and Yuan, 2021), carried out by negative regulators of the pathway, tended to break the accumulation of anthocyanins, once activated. MYB-ATV was expressed more than the other repressors, but, being not functional in both genotypes for the presence of the *atv* allele (Cao et al., 2017; Colanero et al., 2018), its role may have been replaced by MYB-ATV-like and THM27, which showed a similar pattern (Figure 2B).

With ripening, the transcription of the anthocyanin structural genes decreased in *Aft/atv* fruits, highlighting, besides the action of the repressors, an overall reduced activation exerted by light on the metabolic pathway under study, proved by the lower expression of *HY5* and of some activator genes (Figures 4A, B, D). In *Aft/atv/hp2* exocarp, on the other hand, the reduced expression of *HY5* at MR may have been counterbalanced by its stabilization at the protein level, since some of its targets, including most anthocyanin regulatory genes, still showed high transcription (Figure 4B). Nevertheless, also in this genotype many structural genes at MR were expressed less in exocarp than at MG: since many negative regulators were expressed in *Aft/atv/hp2* more than in *Aft/atv* exocarp, stronger feedback repression mechanisms may have contributed to slow down the pathway.

WRKY44 and *GL2*, similarly to the other regulators of the process, were expressed almost exclusively in the exocarps (Figures 2B, 4B), and, differently from earlier hypotheses (Qiu et al., 2019), they also appeared to be under HY5 control, being significantly more expressed in *Aft/atv/hp2* fruits. WRKY TFs involved in anthocyanidin and proanthocyanidin synthesis and homologs of *Arabidopsis* TTG2, which regulates the seed coat tannin accumulation (Gonzalez et al., 2016), have been identified in different species: they could interact with the MBW complex to increase its activity toward specific targets (Lloyd et al., 2017; Amato et al., 2019). The homolog of WRKY44 in kiwifruit, for example, has been recently shown to activate the promoters of *F3′H* and *F3′5′H* (Supplementary Figure S1), regulating important branch points of the pathway (Peng et al., 2020). *WRKY44*, like *AN2-like* and *AN1*, is not expressed in wild-type tomato fruit (Supplementary Figure S5); thus, its transcription in anthocyanin-enriched tomatoes suggests a possible role as an additional activator of the pathway. GL2 is a leucine-zipper TF still not well characterized in tomato and homolog to *Arabidopsis* GL2, which is involved in trichome development and inhibition of anthocyanin synthesis (Chen and Wang, 2019). Actually, some MG *Aft/atv/hp2* fruits showed slightly longer trichomes on their skin (Figure 1C): therefore, tomato GL2 might play functions close to its *Arabidopsis* counterpart, also inhibiting the anthocyanin pathway.

ERF.G3-like behaved differently from all the other TFs analyzed. Its expression was absent in MG fruits and strongly increased with ripening, particularly in *Aft/atv/hp2* fruits (Figure 4B). Recent studies have indicated that *ERF.G3-like* is transcriptionally activated by the master ripening regulator RIN and particularly expressed in tomato mesocarp (You et al., 2024); it

has been also associated with activation of ethylene synthesis and expression of some flavonoid *EBGs* (Li et al., 2020). In our system, we observed activation of *ERF.G3-like* expression in MR mesocarps, besides exocarps, but without concomitant activation of the *EBGs* (Figures 4A, B). Ethylene has been recently found to inhibit anthocyanin synthesis in *Aft*/*atv* tomatoes by specifically repressing the transcription of *AN2-like* and of several structural genes (Xu et al., 2022). Remarkably, multiple EREs were found in the promoter of *AN2-like*, as well as in *AN1* and *AN11* (Figure 5): as a consequence, in *Aft/atv* fruits a possible repressor activity of ethylene on *AN2-like* expression at MR, possibly mediated by ERF.G3-like, could not be excluded. The higher expression of this gene in *Aft/atv/hp2* fruits may also indicate that *ERF.G3-like* is a target of HY5, as other ethylene signaling genes (Wang et al., 2021).

Components of the light signaling pathway may be controlled by regulatory loops during tomato ripening

The anthocyanin biosynthetic pathway may be controlled by the light signaling factors not only at the transcriptional level but also through physical interactions of these factors with the MBW complexes or single components of them, altering their activities. In particular, degradation of AN2-like, whose homologs in other species can be direct targets of the COP1/SPA system (Li et al., 2012; Maier et al., 2013), may not be excluded in *Aft*/*atv* fruits, where both *COP1 homolog* and *COP1-like X1 isoform* resulted expressed, particularly in mesocarp where also the expression of *AN2-like* was lower. However, degradation of AN2-like mediated by the COP1/SPA system could not occur in *Aft*/*atv/hp2* fruits due to the mutation of *DET1* which impaired COP1 activity (Lau and Deng, 2012; Cañibano et al., 2021), but these fruits did not show pigmentation in the mesocarp, as well as the *Aft*/*atv* fruits. Other mechanisms must therefore be hypothesized.

RUP and the BBXs may have indirectly affected the anthocyanin pathway by altering the HY5 levels (Xu, 2020; Zhang et al., 2021). *RUP* expression was very similar in exocarp in the two lines at MG, and then it strongly increased at MR, but only in *Aft*/*atv/hp2* fruit (Figure 4D). RUP is a UV-B light signaling inhibitor, whose expression can be induced by UV light via UVR8 and by HY5 (Zhang et al., 2021), and is produced to revert the active UVR8 monomer to the inactive homodimer (Heijde and Ulm, 2013), contributing, in this way, to also control *HY5* expression. In lettuce, an inhibitory action on the anthocyanin pathway has been recently demonstrated (Yamashita et al., 2023). With the quality of light not changed from MG to MR stages, the strong increase of *RUP* expression in *Aft*/*atv/hp2* fruits at MR may have been induced only by the stabilization of HY5, thus creating an inhibitory loop on further *HY5* expression and anthocyanin production, counteracting, at least in part, the positive effects on the pathway of the stabilization of HY5.

The BBX encoding genes showed expression levels not very different in the two genotypes (Figures 2D, 4D), with only a slight tendency, statistically not significant, of higher mRNA levels in *Aft*/*atv/hp2* MR fruits. Recently, in tomato, the redundant roles of BBX20 and BBX21 in photomorphogenesis have been hypothesized, and the complex produced by BBX20 or BBX21 with HY5 under UV-B has resulted able to activate the expression of *HY5*. However, HY5 protein, in turn, would outcompete BBX20 and BBX21 for binding to its promoter, thus producing an autoregulatory negative feedback loop attenuating its transcription (Yang et al., 2022). In our system, *BBX20* and *BBX21* were both transcribed at MG, and at MR the expression of *BBX20* increased a lot: this could imply a higher inhibitory loop on *HY5* transcription in MR, thus contributing to reduce the *HY5* expression. In *Arabidopsis thaliana*, BBX22 and BBX24 act in both cases in association with HY5, but the first is an activator and the second an inhibitor of the anthocyanin pathway (Jiang et al., 2012; Job et al., 2018; Liu et al., 2022b). The expression patterns of these two genes in MG and MR fruits were overall quite similar in the two lines: thus, they would not seem to be targets of HY5 at the transcriptional level. Furthermore, they were expressed in both fruit exocarps and mesocarps; then, a specific role in the pigmentation patterns of the fruits cannot be inferred by these experimental data.

Anthocyanins accumulated in the fruit exocarp prevent *AN1* transcription leading to acyanic mesocarps

Although *Aft/atv/hp2* fruits showed an exaggerated photomorphogenic phenotype under light, they did not synthesize anthocyanins underneath the exocarp, like the *Aft/atv* fruits. Thus, in both lines, a factor necessary to inducing the pathway should have been missed. *HY5* expression in mesocarp was lower than in exocarp and remained quite stable from MG to MR in both lines. The transcription of *HY5* is mainly activated by UV light through the UVR8/COP1 complex (Chen et al., 2022), whereas its protein stability is controlled by red and blue light photoreceptors via COP1 removal (Texteira, 2020; Bianchetti et al., 2022; Yan et al., 2023), less effective in the presence of the *DET1*/*hp2* mutation. In our system, all the genes encoding photoreceptors or their apoproteins were expressed at MG; then, with ripening, a global reduction in their expressions was observed in both lines (Figures 2C, 4C), and this could have in parallel affected HY5 levels.

In each genotype, at both MG and MR, most of the photoreceptor genes were less expressed in mesocarp than in exocarp (Figures 2C, 4C). If the anthocyanins already present in the exocarp had absorbed specific radiations more than others, the same should have been perceived less in the underneath tissues. UV-A and UV-B are the wavelengths generally mainly filtered by anthocyanins, but also blue photons can be effectively absorbed (Landi et al., 2021). The artificial lightening system used (Supplementary Figure S7) had a spectrum very similar to sunlight; thus, the anthocyanins in the fruit exocarps should have mainly filtered UV and blue light, which are the radiations perceived by UVR8 and cryptochromes: interestingly, the relative encoding genes resulted the more dampened in mesocarp. The reduction of *UVR8* expression in mesocarp may have particularly contributed to inhibit the early steps of the pathway, since flavonoid genes, including *MYB12*, *CHS1*, and *CHS2*, are mainly induced in tomato by UV via UVR8 (Liu et al., 2020). Blue light has been involved in inducing anthocyanin accumulation in strawberry,

pepper, and blueberry fruits (Kadomura-Ishikawa et al., 2013; Liu et al., 2022a; Wei et al., 2023), and in tomato flavonoids, chlorophylls and carotenoids are strongly affected by CRY1 and CRY2 activities (Giliberto et al., 2005; Fantini et al., 2019). A reduction in UV perception by UVR8 and in blue-light perception by cryptochromes may have thus strongly affected anthocyanin synthesis in mesocarps through reduction of *HY5* gene transcription and HY5 protein stability, and this should have been more severe where anthocyanin concentrations in exocarps were higher, that was in *Aft/atv/hp2* fruits. Confirming that, all the photoreceptor genes reduced their expressions from exocarp to mesocarp more in *Aft/atv/hp2* fruits than in *Aft/atv* (Supplementary Figure S4, columns B, C, E, F): a light hypersensitivity, such as the one shown by the *hp2* mutants, was thus not more permissive since the higher anthocyanins levels accumulated in the exocarp produced an even thicker shield for light penetration inside the fruit, neutralizing the stabilization of HY5 due to the failure of COP1-mediated turnover.

The lack of anthocyanins in mesocarp might thus indicate that AN2-like, insufficiently induced by HY5, could not have reached a threshold necessary to produce the MBW1 complex in the amounts needed to activate *AN1* transcription. The same direct activation exerted by HY5 and other light-responsive factors on the transcription of *AN1* and *AN11* might have been lost or highly

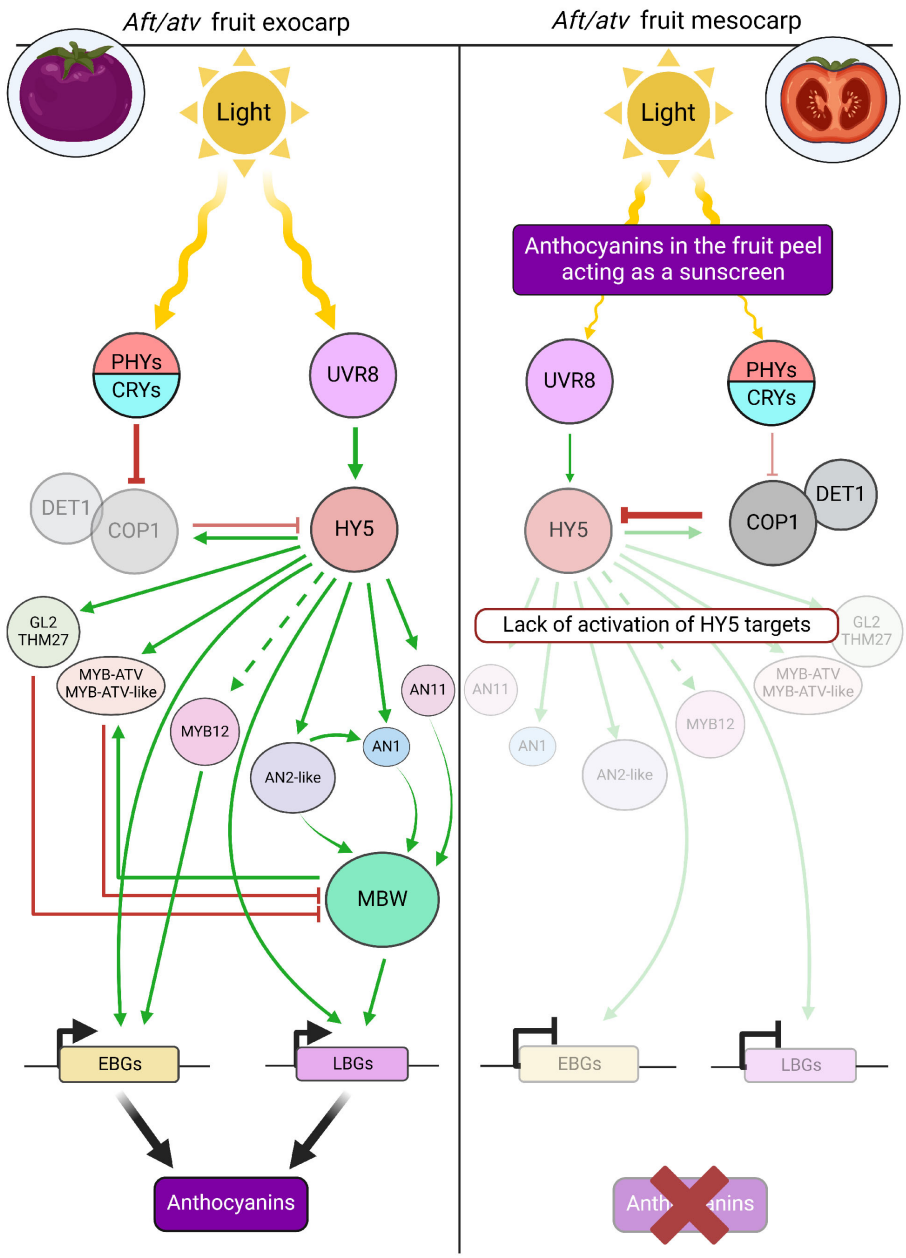

FIGURE 6
Final scheme summarizing the main elements which under light regulate anthocyanin pigmentation in the exocarp (left) and mesocarp (right) of *Aft/atv* tomato fruits.

reduced in mesocarp because of the filter exerted by the anthocyanin light screen. As a consequence, *AN1* expression resulted negligible in mesocarp (Figures 2B, 4B) and thus ineffective to produce the MBW2 complex necessary to activate the expression of the *LBGs*: this would have finally impaired the synthesis of anthocyanins inside the fruit.

To circumvent this bottleneck, an adequate increase of the transcription rate of *AN2-like* or *AN1* in mesocarp would be necessary. Low temperature may represent a trigger for anthocyanin synthesis in fruits of several species (Zhang et al., 2011; Gao-Takai et al., 2019; Xue et al., 2021; Dai et al., 2022), increasing the expression of R2R3-MYB or bHLH TF-encoding genes and/or improving the binding ability of bHLH and MYB inside the MBW complexes (Sheerin and Hiltbrunner, 2017). Furthermore, HY5 levels under light are positively regulated by low temperature, both transcriptionally, via a CBF- and ABA-independent pathway, and posttranslationally, via protein stabilization through nuclear depletion of COP1 (Catalá et al., 2011). Previous studies indicated that cold could activate both *AN2* and *AN1* expressions in tomato plants (Kiferle et al., 2015), and, as observed in the present work, it may act on the *R2R3-MYB* gene transcription through HY5 but also directly on *AN1* and *AN11* expression being several LTR elements present in their promoters (Figure 5). Expositions to low temperature may therefore effectively integrate the light stimulus, finally leading to purple tomatoes containing anthocyanins in all the pericarp.

In conclusion, the analyses carried out in the present study, whose main results are summarized in Figure 6, indicated that i) the anthocyanin biosynthetic pathway could be activated by light in the exocarp of *Aft/atv* fruits, reflecting the role of flavonoids in tomato fruit photoprotection; ii) between photoreceptors and anthocyanin production, a signaling cascade based on HY5 activated, either directly or indirectly, anthocyanin regulatory and biosynthetic genes; iii) a combinatorial effect of light-mediated signals with AN2-like may have been necessary to guide *AN1* expression as well as transcription of many structural genes; iv) the light penetrating inside the fruit was qualitatively/quantitatively different from radiations incident on the skin, as a result of the anthocyanins accumulated in the exocarp which shaded the inner fruit tissues; v) *HY5* expression in mesocarp was low because of the scarce activation exerted by the photoreceptors on its expression and protein stability, and inhibitory loops on its transcription produced by BBX factors and RUP proteins may not be excluded; and vi) in the absence of other inducing factors, e.g., low temperatures, the anthocyanin regulatory genes were very poorly transcribed in tomato mesocarp, starting from the R2R3-MYB activators *MYB12* and *AN2-like* till the bHLH gene *AN1*, whose absence is the primary cause of the silence of both *EBGs* and *LBGs*.

Data availability statement

The data presented in the study are deposited in the Gene Expression Omnibus repository, accession number GSE282571.

Author contributions

SG: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Supervision, Writing – original draft, Writing – review & editing. JM: Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. PP: Funding acquisition, Resources, Supervision, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The author(s) declare that no Generative AI was used in the creation of this manuscript.

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The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphgy.2024.1507833/full#supplementary-material

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