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What's behind Purple Tomatoes? Insight into the Mechanisms of Anthocyanin Synthesis in Tomato Fruits[OPEN]

Sara Colanero, Pierdomenico Perata,[1,2] and Silvia Gonzali

PlantLab, Institute of Life Sciences, Scuola Superiore Sant'Anna, 56127 Pisa, Italy

ORCID IDs: 0000-0003-1784-6187 (S.C.); 0000-0001-9444-0610 (P.P.); 0000-0003-3671-4999 (S.G.).

The term "anthocyanins" was first used in 1835 by L.C. Marquart to indicate the blue pigments of flowers (Shibata et al., 1919). In the early 20th century, these pigments, whose colors range from red to purple to blue, were first isolated and chemically characterized by Richard Willstatter (Robinson and Robinson, 1931). A wide range of different anthocyanins have subsequently been extracted from flowers, fruits, seeds, roots, and leaves, thus increasing the size of this subclass of polyphenolic compounds belonging to the larger class of flavonoids (Koes et al., 2005). Anthocyanins are glycosylated, polyhydroxy or polymethoxy molecules, formed by two aromatic rings connected by a C3 bridge to a benzene ring (Fig. 1A). These pigments are water-soluble and mainly accumulate in the vacuole of plant cells: they are odorless and flavorless, although their presence in edible fruits and vegetables gives a slightly astringent taste (Martín et al., 2017). A total of 702 different anthocyanins and 27 anthocyanidins (aglycone forms of anthocyanins) have been classified (Krga and Milenkovic, 2019). All these phenolic compounds vary from each other in the different groups of carbon substitutions on the structural rings (Fig. 1A; Liu et al., 2018).

Anthocyanins play a major role during plant life and development. Initially, they were seen as having just one main function—i.e. their colored pigments attracted pollinating insects and seed-spreading animals (Harborne and Williams, 2000; Hoballah et al., 2007). It then became clear that plants also produce anthocyanins to protect themselves from a wide variety of biotic and abiotic stimuli (Chalker-Scott, 1999). When plants are exposed to different types of stresses, such as high light, heavy metals, salty soils, drought, high/low temperatures, as well as pathogen or insect attacks, there is typically an increase in reactive oxygen species (ROS) production, which may then be counterbalanced through the generation of antioxidant molecules

(Sharma et al., 2019). Anthocyanins, which primarily accumulate in the subepidermal cell layers of vegetative tissues, act as a photoprotective light screen, by absorbing potentially damaging UV-B radiation (Gould, 2004). They can also act as antioxidant compounds through different chemical mechanisms, such as releasing hydrogen atoms from the hydroxyl groups or as metal-chelating agents, thus efficiently scavenging free radicals and ROS (Gould, 2004). Through the same mechanisms, anthocyanins can delay senescence processes. In deciduous species, for example, they can protect senescing leaves by acting as ROS scavengers and as a light screen against photoinhibitory irradiances that can damage the photosynthetic machinery while it is losing its photoprotective capacity (Hoch et al., 2001).

ADVANCES

- Tomato is an ideal target for studies on enhancing the biological value of vegetables in terms of bioactive polyphenolic compounds, whose levels are suboptimal, particularly due to the absence of anthocyanins.

- Anthocyanins are increasingly being investigated as health-beneficial phytochemicals in preventive medicine research.

- Anthocyanin-enriched tomato fruits have been produced using genetic engineering or breeding approaches.

- Recent studies have indicated how tomato has the genetic capacity to produce anthocyanins, but, in wild-type tomatoes, the pathway is switched off by mutations in regulatory elements.

- Sustainable expression of a suitable R2R3 MYB transcription factor represents the minimum requirement to switch on the anthocyanin pathway in tomato fruits.


Figure 1. Anthocyanin biosynthesis in higher plants. A, Chemical structure of the general anthocyanin molecule and main anthocyanin pigments synthesized in plants, according to the different nature of the R lateral groups. B, Anthocyanin biosynthetic pathway. Enzyme abbreviations: PAL, Phenylalanine ammonia-lyase; C4H, cinnamate-4-hydroxylase; 4CL, 4-coumaroyl:CoA-ligase; CHS, chalcone synthase; F3H, flavanone 3-hydroxylase; F3′H, flavonoid 3′-hydroxylase; F3′5′H, flavonoid 3′5′-hydroxylase; ANS, anthocyanin synthase; UFGT, anthocyanidin 3-O-glucosyltransferase. In the final part of the biosynthetic way, from dihydroflavonols to anthocyanins, the pathway which is predominant in tomato plants is highlighted.
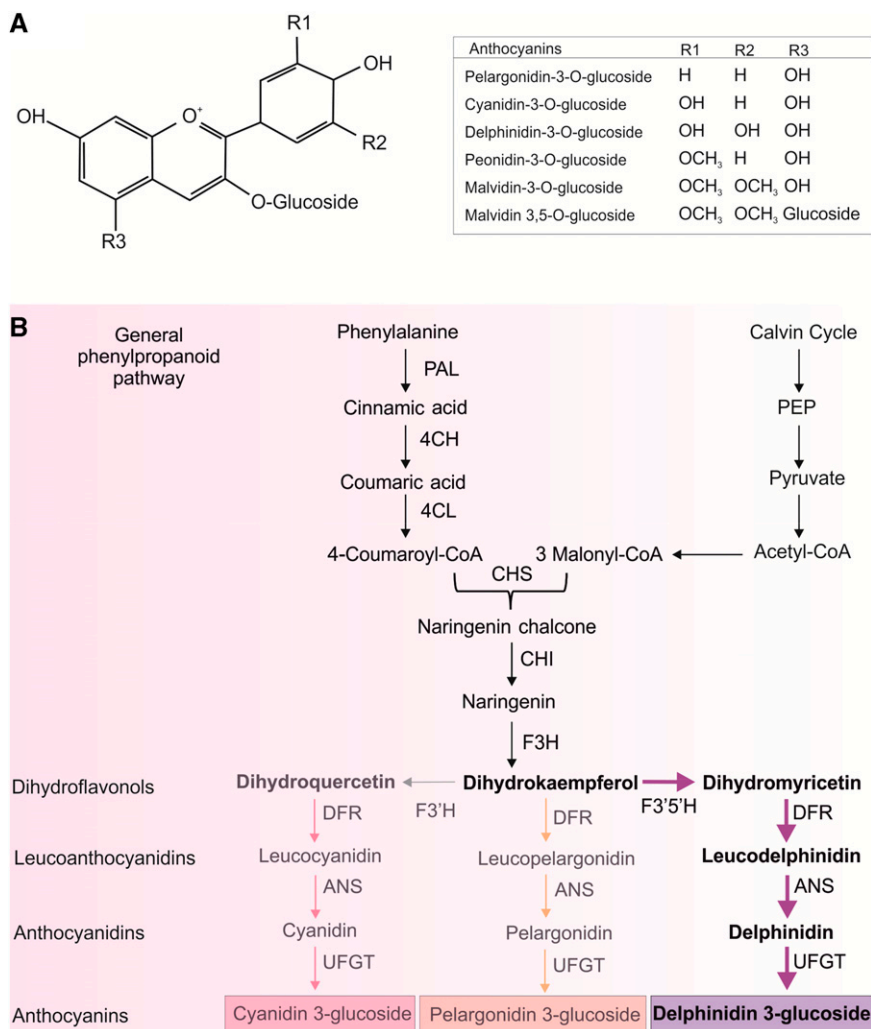
Anthocyanins are beneficial metabolites not only for plants; they are widespread in edible fruits and vegetables and thanks to their antioxidant and anti-inflammatory actions they can provide pharmacological activity (Li et al., 2017; Liu et al., 2017). Integrating high phenolic content foods into the human diet is believed to help prevent and control diseases such as cardiovascular disorders (Mink et al., 2007; Mauray et al., 2010; Cassidy et al., 2013), diabetes and obesity (Muraki et al., 2013; Wang et al., 2013), and cancer (Li et al., 2017). Anthocyanins may also have a neuroprotective activity (Youdim et al., 2004), and promising results have been demonstrated in animal models (Wang et al., 2012a, 2012b). Plant research has thus moved toward understanding the regulatory mechanisms of anthocyanin synthesis to produce anthocyanin-enriched versions of horticultural crops so as to increase the daily uptake of these health-promoting compounds (Martin et al., 2011).

Because it is one of the most consumed vegetable products in the world, tomato (*Solanum lycopersicum*) has been extensively used to achieve this goal. Unlike other *Solanaceae* such as eggplant (*Solanum melongena*) or pepper (*Capsicum* spp.), tomato fruits do not normally accumulate anthocyanins due to the incomplete activation of the flavonoid pathway (Povero et al., 2011). To increase their content, genetic engineering methods (Butelli et al., 2008) or genotype selection arising from natural breeding (Mes et al., 2008; Gonzali et al., 2009) have thus been exploited with excellent results. This review summarizes and discusses recent research into breeding purple varieties of tomato.

REGULATION OF THE ANTHOCYANIN BIOSYNTHETIC PATHWAY

Anthocyanin biosynthesis is well characterized in higher plants and consists of a strongly conserved pathway (Holton and Cornish, 1995; Fig. 1B). It shares the first part of the required biosynthetic enzymes with other flavonoids, such as flavonols and flavanones, and represents a branch of the general phenylpropanoid pathway, whose main precursor is Phe, which itself

comes from the shikimic acid pathway (Rasmussen and Dixon, 1999). From the condensation of one molecule of 4-coumaroyl CoA (from Phe) and three of malonyl CoA (from fatty acid metabolism), through the combined consecutive actions of three different enzymes—chalcone synthase, chalcone isomerase (CHI), and flavanone 3-hydroxylase the dihydroflavonol dihydrokaempferol is produced. Acting in the first part of the biosynthetic pathway, the genes that encode these enzymes are named *early biosynthetic genes* (*EBGs*). Dihydrokaempferol can then be hydroxylated, through the action of flavonoid 3′-hydroxylase or flavonoid 3′-5′-hydroxylase (F3′5′H), to produce the other two dihydroflavonols, dihydroquercetin and dihydromyricetin, respectively. Downstream, the dihydroflavonols are reduced by dihydroflavonol 4-reductase (DFR) into different leucoanthocyanidins, which are converted into the corresponding anthocyanidins (leucopelargonidin, leucocyanidin, and leucodelphynidin) by the action of anthocyanin synthase (also called LDOX; Holton and Cornish, 1995; Springob et al., 2003). The colorless anthocyanidins are then glycosylated at C3, giving rise to the corresponding colored anthocyanin compounds (pelargonidin, cyanidin, and delphinidin), which are chemically stable and water-soluble (Sasaki et al., 2014). There are several forms of additional glycosylation, as well as acylation, which produce many different molecules. In addition to the three compounds mentioned above, other common anthocyanins found in plants are peonidin and petunidin (from methylation at the 3′ hydroxyl group of cyanidin and delphinidin, respectively), and malvidin (from methylation at the 3′ and 5′ hydroxyl groups of delphinidin). The final step of the pathway is the transport into the vacuole, through the action of glutathione *S*-transferase (GST) or putative anthocyanin transporter (Gonzali et al., 2009). The genes encoding the enzymes acting downstream of the dihydroflavonols are called *late biosynthetic genes* (*LBGs*), and are those specifically regulated for anthocyanin production.

Many plants exhibit a limited range of anthocyanin colors due to the lack of an essential gene or to the substrate specificity of a biosynthetic enzyme. Although in most cases DFR enzymes can utilize all three dihydroflavonols as substrates, in some species they show substrate specificity due to minor changes in their molecular structure (Johnson et al., 2001). Similar to petunia (*Petunia x hybrida*) and other *Solanaceae*, in tomato the action of DFR is mainly carried out on dihydromyricetin. The delphinidin-type anthocyanins are thus the most abundant in this species (Fig. 1B; Bovy et al., 2007).

Complex regulatory mechanisms induced by developmental and environmental stimuli activate and/or repress the anthocyanin biosynthetic pathway (Albert et al., 2014). A central role is played by specific transcription factors (TFs), which, induced or repressed by different signals, can act individually or in multiprotein complexes to activate the transcription of *EBGs* and/or *LBGs*, thus modulating the synthesis of anthocyanins to
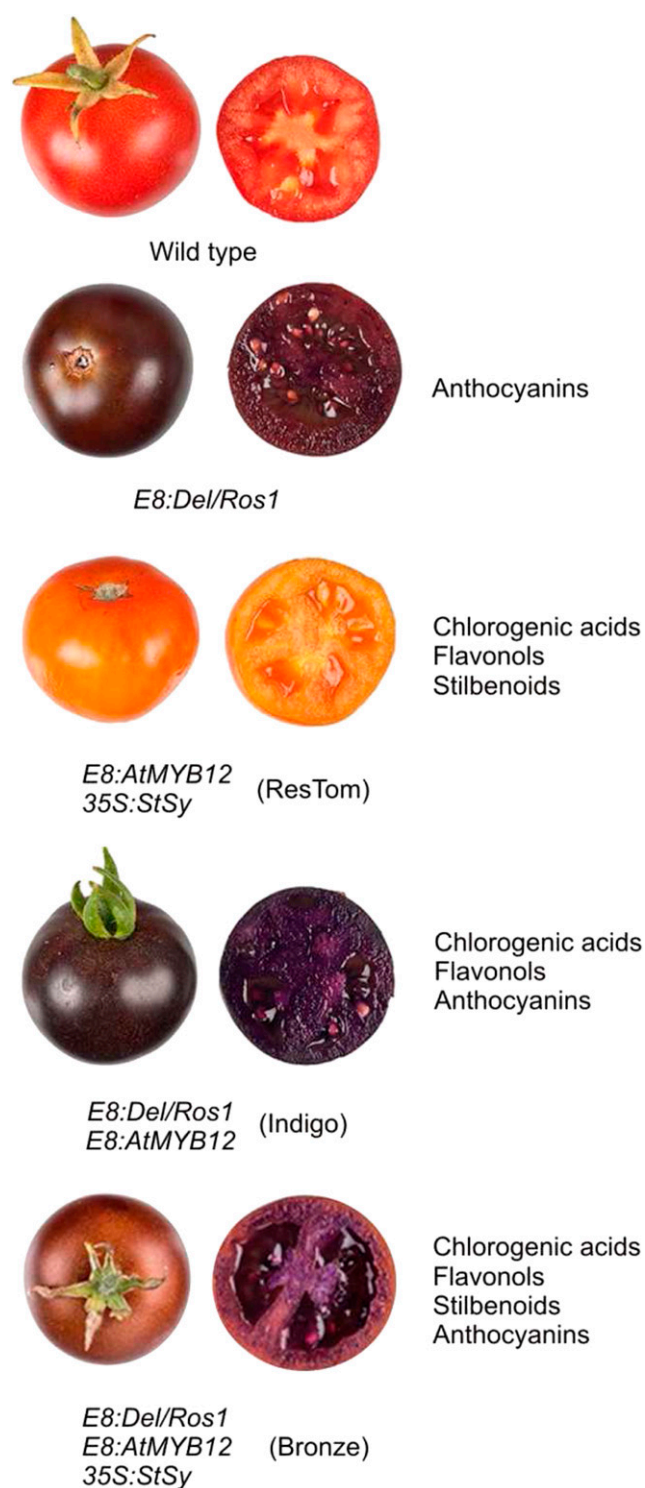

Figure 2. Purple tomatoes produced by genetic engineering. The main classes of polyphenols synthesized in each tomato line are reported. Abbreviations: *AtMYB12*, *A. thaliana MYB12*; *StSy*, *V. vinifera Stilbene Synthase*. The photographs of wild-type, *Del/Ros1*, ResTom, and Indigo fruits are from Zhang et al. (2015; CC BY 4.0, http://creativecommons.org/licenses/by/4.0/). The photographs of Bronze tomatoes are from Scarano et al. (2018; CC BY 4.0, https://creativecommons.org/licenses/by/4.0/). No changes were made to the photographs.

the different needs of the plant. Among these TFs, R2R3 MYBs cooperate with basic Helix–Loop–Helix (bHLH) and WD-repeat (WDR) proteins to form ternary MYB–bHLH–WDR (MBW) complexes (Koes et al., 2005; Ramsay and Glover, 2005; Albert et al., 2014; Liu et al., 2015; Montefiori et al., 2015; Xu et al., 2015). Some of the TFs are constitutively expressed in plant cells, whereas others require transcriptional activation. The R2R3 MYB factors are induced by the triggering signals (developmental or environmental) and arrange themselves into the MBW complex, by binding constitutively expressed or transcriptionally induced bHLH and WDR proteins (Albert et al., 2014). The MBW complex is the key regulator of the pathway because it triggers the transcription of biosynthetic genes, *LBGs* in particular, and thus the final part of the biosynthetic pathway (Koes et al., 2005). It can also activate the expression of negative regulators, such as R3 MYB proteins, which inhibit anthocyanin production through a negative feedback mechanism (Koes et al., 2005; Albert et al., 2014; Colanero et al., 2018). Several R2R3 MYB proteins, bHLH, WDR, and R3 MYBs, have recently been characterized in plants (Koes et al., 2005; Hichri et al., 2011; Zhang et al., 2019), thus providing an increasingly comprehensive picture of how the regulation of the anthocyanin biosynthetic pathway works.

SWITCHING ON THE ANTHOCYANIN PATHWAY IN TOMATO FRUITS

Tomato was imported to Europe in the 16th century from central and southwestern America. It was initially used as an ornamental plant and then gradually became an economically important crop for human consumption (Peralta and Spooner, 2007). During domestication, much of the genetic variability was lost (Bai and Lindhout, 2007) and the selection toward new productivity traits had a negative impact on some other important features. The size and color of the fruit also changed over time (Tanksley, 2004).

Tomatoes provide humans with key nutrients in their daily diet such as lycopene, the most abundant carotenoid in the ripe fruit, which has a strong antioxidant activity that is beneficial in the prevention of a wide range of diseases (Martí et al., 2016; Mozos et al., 2018). Moreover, tomatoes contain a smaller amount of other carotenoids and a high amount of soluble sugars, organic acids, amino acids, vitamins, and volatile compounds, which all affect the characteristic flavor (Tieman et al., 2017; Wang and Seymour, 2017).

Anthocyanins are generally not found in tomatoes, whereas other flavonoids, such as flavonols (mainly quercetin, myricetin, and kaempferol) and flavanones (narigenin), are detectable (Scarano et al., 2018). There is thus growing interest in expanding the repertoire of health-promoting compounds in tomato fruit to further enhance its already high nutraceutical potential. For this reason, molecular biologists and plant breeders are producing tomato lines with increased anthocyanin
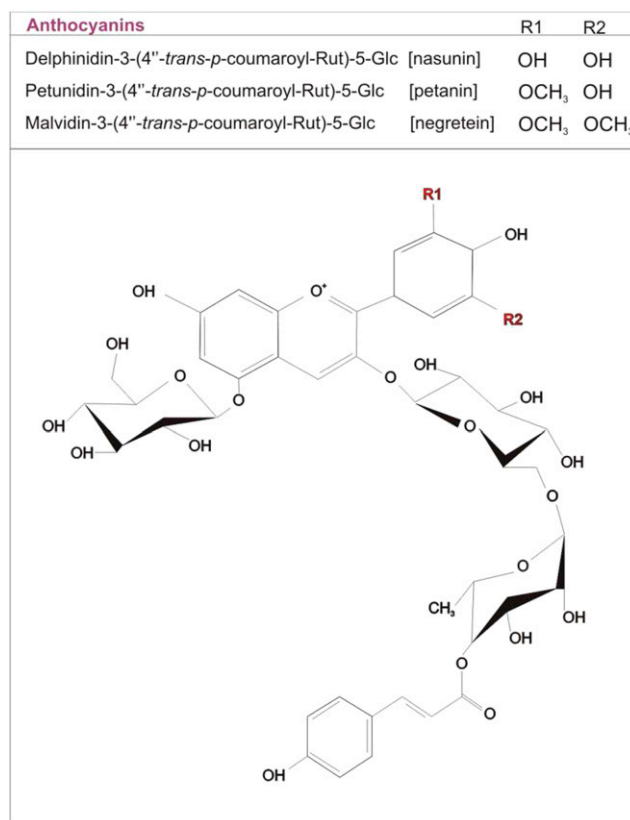

Anthocyanins		R1	R2
Delphinidin-3-(4"-*trans-p*-coumaroyl-Rut)-5-Glc	[nasunin]	OH	OH
Petunidin-3-(4"-*trans-p*-coumaroyl-Rut)-5-Glc	[petanin]	OCH₃	OH
Malvidin-3-(4"-*trans-p*-coumaroyl-Rut)-5-Glc	[negretein]	OCH₃	OCH₃

Figure 3. Main anthocyanins synthesized in purple tomato fruits. Petanin and delphinidin-3–(4''–trans–*p*–coumaroyl)-Rut-5-Glc are the most abundant anthocyanins identified in *Del/Ros1* tomato fruits. Petanin and malvidin-3-(4''–trans–*p*–coumaroyl)-Rut-5-Glc are the most abundant molecules identified in *Aft/Aft atv/atv* tomato fruits. The chemical structure was redrawn from Blando et al. (2019).

content in their fruits (Butelli et al., 2008; Mes et al., 2008; Gonzali et al., 2009). In the next subsections we will discuss how this has been achieved.

Metabolic Engineering Studies Led To the Production of Purple Tomato Fruits

Initial attempts to increase the flavonoid content in tomato fruits started in the early 2000s with the overexpression of structural genes encoding enzymes of the pathway. Given the low level of expression of the gene encoding the CHI enzyme compared to the other *EBGs*, the petunia *chalcone isomerase* gene was overexpressed in tomato plants to enhance the basal level of flavonoids (Muir et al., 2001). The transgenic plants showed an increase of up to 78-fold in fruit peel flavonols, mainly due to the accumulation of quercetin glycoside and lower amounts of kaempferol glycoside (Muir et al., 2001). No anthocyanins were detected and the whole biosynthetic pathway appeared to be switched off in the fruit flesh (Muir et al., 2001). Using a similar approach, Schijlen et al. (2006) generated transgenic tomato plants overexpressing structural flavonoid genes
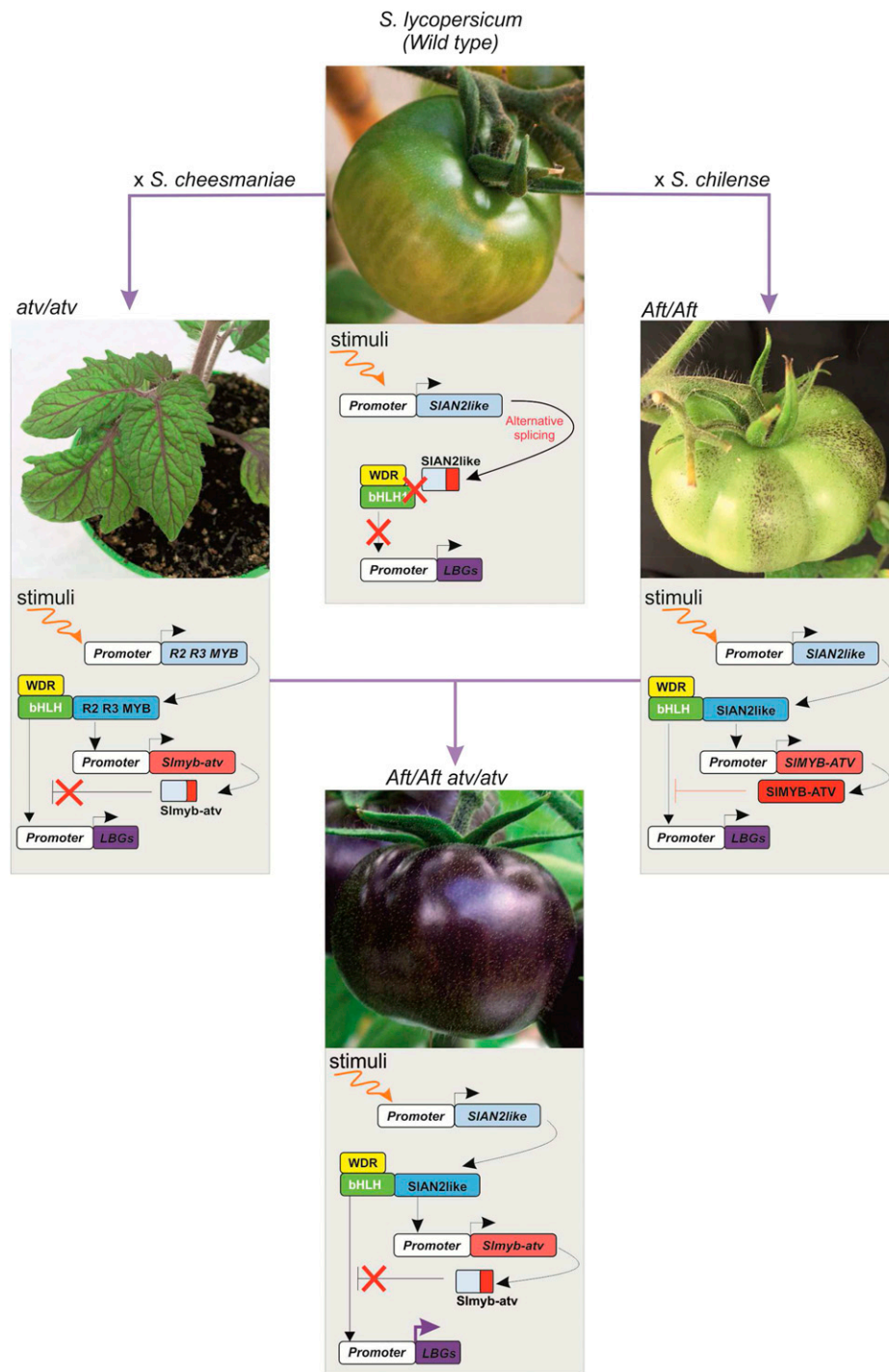
Figure 4. Breeding anthocyanin-enriched tomato lines for the production of high-anthocyanin tomato fruits. The main regulation of the anthocyanin pathway is summarized under the picture of each tomato line. In the wild-type tomato fruit (top), the R2R3 MYB TF encoding *SlAN2like* gene is activated by an environmental signal (e.g. high amount of light), but, due to an alternative splicing mutation, its corresponding protein is truncated and nonfunctional. As a consequence, it cannot interact with a bHLH-partner protein and thus participate in the MBW complex that activates the transcription of different biosynthetic genes in the flavonoid pathway, including the *LBGs*, which are essential for the anthocyanin production. Anthocyanins are thus not produced. In the fruits of the *atv* tomato lines (left), the same situation described above is observed. However, in other organs of the *atv* plant, for example in the vegetative tissues, anthocyanin synthesis (activated by a suitable stimulus on the promoter of an R2R3 MYB TF encoding gene—e.g. *SlAN2*) is higher than in the wild-type plants because a repressor of the pathway, an R3 MYB factor encoded by the *SlMYB-ATV* gene, is mutated. The corresponding protein (slmyb-atv) is truncated and no longer able to repress the action of

Table 1. *Genes encoding the main regulators of the tomato anthocyanin biosynthetic pathway*

Gene Code	Name	Function	References
Solyc10g086250	*SlAN2, SlMYB75*	R2R3 MYB	Povero et al., 2011; Myers, 2012; Kiferle et al., 2015
Solyc10g086260	*SlANT1, anthocyanin1*	R2R3 MYB	Mathews et al., 2003; Sapir et al., 2008; Povero et al., 2011; Schreiber et al., 2012; Kiferle et al., 2015
Solyc10g086270	*SlANT1like, SlMYB28*	R2R3 MYB	Kiferle et al., 2015
Solyc10g086290	*SlAN2like, SlMYB114*	R2R3 MYB	Kiferle et al., 2015; Cao et al., 2017; Qiu et al., 2019; Yan et al., 2019; Colanero et al., 2020; Sun et al., 2020
Solyc09g065100	*SlAN1, AH, SlTT8*	bHLH	Kiferle et al., 2015; Qiu et al., 2016
Solyc08g081140	*SlJAF13, SlGL3*	bHLH	Nukumizu et al., 2013; Kiferle et al., 2015
Solyc03g097340	*SlAN11*	WDR	Kiferle et al., 2015; Gao et al., 2018
Solyc07g052490	*SlMYB-ATV*	R3 MYB	Cao et al., 2017; Colanero et al., 2018; Yan et al., 2019; Sun et al., 2020

from different plant sources. High levels of stilbenes, deoxychalcones, and flavones were obtained in these plants, in relation to the structural gene introduced (Schijlen et al., 2006). Although the overexpression of the *EBGs* did not affect the anthocyanin branch of the biosynthetic pathway, it strongly increased the total antioxidant activity measured in the fruit peel extracts (Schijlen et al., 2006). Such studies indicated that to trigger anthocyanin synthesis, increasing the expression of the *EBGs* is not enough. Specific regulatory TFs are necessary to trigger *LBG* expression.

Bovy et al. (2002) tried to overexpress in tomato two anthocyanin biosynthetic regulatory genes of maize (*Zea mays*), the MYB gene *C1* and the bHLH gene *LC*, which in other species ectopically activated anthocyanin synthesis. The transgenic tomatoes showed high level of flavonols in both the peel and flesh (Bovy et al., 2002). These two regulatory genes were thus sufficient to activate the flavonoid pathway in the flesh of the fruits. However, no change in the anthocyanin content was measured (Bovy et al., 2002). This was mainly due to the inability of the two maize TFs to activate the *F3'5'H* structural gene, which is necessary to produce dihydromyricetin, which, as explained above, represents the main dihydroflavonol of the anthocyanin biosynthetic pathway in tomato (Fig. 1B). The overexpression of other MYB-type TFs, both endogenous, such as *SlANT1* (Mathews et al., 2003), and heterologous, such as *AtPAP1* (Zuluaga et al., 2008), led to the first partial successes with tomatoes characterized by an anthocyanin-spotted fruit peel. Finally, anthocyanin-enriched tomatoes were produced when Butelli et al. (2008) overexpressed the regulatory genes *Delila*

(*Del*; *bHLH*) and *Rosea1* (*Ros1*; *R2R3 MYB*) from snapdragon (*Antirrhinum majus*) under the control of the tomato fruit-specific *E8* promoter, resulting in completely purple fruits not only in the peel but also in the flesh (Fig. 2). Del and Ros1 were able to activate not only *LBGs* but also *EBGs*, such as *Phe ammonia-lyase* and *CHI* (Butelli et al., 2008), strongly promoting flavonoid biosynthesis. The anthocyanin content was comparable to the levels found in blackberries and blueberries (>2 mg g^{-1} fresh weight; Butelli et al., 2008). Seven different compounds were identified, with delphinidin-3-(4''–trans–p–coumaroyl)-Rut-5-Glc and petunidin-3-(4''–trans–p–coumaroyl)-Rut-5-Glc (petanin) as major molecules (Tohge et al., 2015; Fig. 3). The *Del/Ros1* tomato extracts were used to feed cancer-susceptible *Trp-53*$^{-/-}$ knockout mice, leading to an extended life span, suggesting that the anthocyanin levels contained in these transgenic tomatoes were sufficient to exert a protective role against cancer progression (Butelli et al., 2008). Remarkably, enrichment of anthocyanins significantly extended the shelf life of the purple tomato fruits, likely suppressing processes of overripening and reducing susceptibility to *Botrytis cinerea*, one of the most important postharvest pathogens (Zhang et al., 2013).

Some years later, in a multilevel engineering approach, *Del/Ros1* plants were crossed with a tomato line overexpressing the Arabidopsis (*Arabidopsis thaliana*) *MYB12* TF, known to be able to strongly activate the flavonoid *EBGs* (Luo et al., 2008). The new tomato line was called "Indigo" (Zhang et al., 2015). *AtMYB12* was able to bind in tomato the promoters of genes encoding enzymes of both primary and secondary

Figure 4. (*Continued.*)
the MBW complex on the *LBGs*. Anthocyanin accumulation, therefore, is higher because it is not limited by the feedback repression mechanism exerted by the R3 MYB protein. In the fruits of the *Aft* line (right), the R2R3 MYB TF encoding gene *SlAN2like* is activated by an environmental signal (e.g. high amount of light) and produces a functional protein that interacts with the bHLH and WDR partners of the MBW complex, thus inducing anthocyanin synthesis. This is counterbalanced by the action of the repressor SlMYB-ATV factor, which, inhibiting the action of the MBW complex on the *LBG* promoters, prevents excessive accumulation of the purple pigments. In the fruits of the *Aft/Aft atv/atv* tomato genotype (bottom), the same activation mechanism mediated by the SlAN2like TF described for *Aft* takes place. In this case, however, the *SlMYB-ATV* gene is mutated as in *atv* plants. Therefore, once anthocyanin synthesis has been activated, it cannot be repressed by the R3 MYB protein—thus leading to a large accumulation of anthocyanins that can produce homogeneous purple pigmentation.

BOX 1. Why is *Aft* tomato peel purple-spotted?

In flower pigmentation it is not rare to find anthocyanin accumulation giving rise to specific patterns such as stripes or spots. There are also similar pigmentation models in fruit peels. The *Aft* tomato fruit typically shows purple anthocyanin spots upon exposure to high light. Why is this pigmentation pattern not uniform? Due to the regulatory mechanism, the synthesis of anthocyanins can only occur within cells where all three MBW components are expressed. Similar to some flower pigmentation patterns (Davies et al., 2012), anthocyanin spots in *Aft* peel (Fig. 4) are generally multicellular and are surrounded by cells where anthocyanins are not produced (halo). It is likely that mobile small regulators of the pathway can move from the cells where they have been produced to spread their action in proximal cells. In particular, small inhibitory molecules, such as the R3 MYB factors, can be synthesized in cells producing anthocyanins through the transcriptional activation carried out by the MBW complex on their promoters.

They then move to neighboring cells where they can inhibit anthocyanin synthesis. In their R3 domain, these factors harbor a specific amino acid signature which allows them to specifically bind bHLH proteins. They can thus compete with the R2R3 MYB activators which, in turn, interact with the bHLH partners in the MBW complexes (Albert et al., 2014). Another possible mechanism is the depletion of one of the components of the MBW complex in the cells surrounding the anthocyanin spot: the WDR factors, for example, can move from cell to cell and this mechanism can lead to a loss of pigmentation (Albert et al., 2014). Considering the change in the pigmentation pattern from spotted to more uniform when the *Aft* mutation is associated with the *atv* one (involving an R3 MYB inhibitor of the anthocyanin pathway, which is no longer functional) it seems more likely that the mobile factor in the tomato fruit peel is the small R3 MYB inhibitor ATV.

metabolism strongly promoting flavonoid biosynthesis. The simultaneous presence of this TF with Del and Ros1 thus significantly increased the contents of the main phenylpropanoids (chlorogenic acids, flavonols, and anthocyanins) in Indigo tomatoes in comparison with the previous lines (Zhang et al., 2015; Fig. 2).

Finally, after the same multilevel approach, Scarano et al. (2018) developed a Bronze tomato characterized by three different classes of polyphenols (flavonols, anthocyanins, and stilbenoids), by introducing, in the Indigo line, the *Stilbene Synthase* gene from *Vitis vinifera* (Fig. 2). Using a mouse model for inflammatory bowel disease, Scarano et al. (2018) showed that supplementing the diet with Bronze tomato significantly reduced bowel inflammation more than the high anthocyanin or high stilbene tomatoes, demonstrating synergistic effects of different classes of polyphenols for reducing inflammation.

The above studies clearly indicate how the various metabolic engineering strategies for the enrichment of anthocyanins and other flavonoid compounds have been very successful in tomato and have increased the range of polyphenolic metabolites that can be produced. However, the purple tomato lines thus produced are not currently commercially available mainly due to the public's worries about the safety of genetically modified food.

Genetic Mapping of Loci Associated with High-Anthocyanin Content in Tomato Fruits: from the Breeding of Purple Fruit-Lines to the Understanding of the Genetic Loci Involved

Although cultivated varieties do not synthesize anthocyanins in the fruits, some wild tomato species (e.g. *Solanum chilense*, *Solanum peruvianum*, *Solanum lycopersicoides*) produce green fruits which, upon suitable light conditions, can accumulate anthocyanins in the peel (Bedinger et al., 2011). Some of the related traits have been introgressed into the cultivated species through interspecific crosses, which in some cases occurred spontaneously (Mes et al., 2008; Gonzali et al., 2009; Myers, 2012). New tomato accessions were thus obtained showing anthocyanins in fruit peel, such as *Anthocyanin fruit* (*Aft*; Box 1) and *Aubergine* (*Abg*), or anthocyanin accumulation in vegetative tissues, such as *atroviolacea* (*atv*; Rick et al., 1968, 1994; Georgiev, 1972). These accessions have been used in subsequent breeding programs to further enrich the anthocyanin content

Different crosses between the accessions *Aft* and *atv* gave rise to the double mutant *Aft/Aft atv/atv* (Fig. 4), producing new lines in different tomato backgrounds. One of them, named "Sun Black", was derived from the accessions LA1996 (*Aft*) and LA0797 (*atv*) (Mazzucato et al., 2013). An extensive biochemical characterization was recently performed in this line (Blando et al., 2019). Although the qualitative and quantitative properties of a fruit can be strongly affected by the type of cultivation, the season, and the environment, plants with the same backgrounds of the purple line, only devoid of the two *Aft* and *atv* introgressed regions, were used in this study: this makes the comparison between the purple and the wild-type (WT) tomatoes particularly valuable. The presence of anthocyanins, which are completely absent in WT fruit, represents the most important difference. In the ripe purple fruits the content of these pigments is higher than 1 mg g^{-1} dry weight (DW). The two main compounds identified are petanin (56.6% of the total), and negretein (21.4%) (Fig. 3). Both are produced through the action of decorating enzymes from delphinidin, confirming the prevalence of this specific anthocyanin in the pigment inventory of tomato (Fig. 1B). Anthocyanins represent the most abundant polyphenolic class. However, other flavonoids increase, and rutin (the main flavonol) reaches 0.8 mg g^{-1} DW. The total phenolic content is 8.6 mg g^{-1} DW, and chlorogenic acid (0.6 mg g^{-1} DW) represents the most abundant phenolic acid. The total amount of carotenoids is similar, if not higher, than in WT, with a total content which exceeds 200 µg g^{-1} DW. The carotenoid composition is slightly affected by the genotype: the most abundant are lycopene, β-carotene, lutein, and α-carotene, and in the purple ripe fruit, the content of lutein, α-carotene and, above all, β-carotene is higher than in WT, whereas the amount of lycopene is slightly lower. Also, the content in vitamin C (37.3 mg 100 g^{-1} fresh weight) is higher in the purple than in the WT fruit, likely due to the protective function exerted by the increased polyphenols on the oxidation of ascorbic acid. Finally, the antioxidant capacity of the purple fruit is significantly high, due to the presence of the anthocyanins and the increased content of the other polyphenolic compounds. On the whole, this study clearly indicated how the enrichment of anthocyanins is a real added value of the purple fruit.

of the fruits. The cross between *Aft* and *atv* or between *Abg* and *atv* produced *Aft/Aft atv/atv* and *Abg/− atv/atv*, respectively, which are two new lines with a strong purple anthocyanin pigmentation in the fruit peel (Mes et al., 2008; Gonzali et al., 2009). The more stable *Aft/Aft atv/atv* genetic combinations were also patented as nongenetically modified purple tomatoes and commercialized under different brands (Myers, 2012; Mazzucato et al., 2013). The strong purple phenotype that these lines can exhibit is due to the synergistic combination of the *Aft* and *atv* traits that allows a sustained activation of many *EBGs* and most of the *LBGs* (Povero et al., 2011). More than 75% of the anthocyanin profile in *Aft/Aft atv/atv* fruits is constituted by petanin and malvidin-3-(4''–trans–p–coumaroyl)-Rut-5-Glc (Fig. 3; Box 2; Blando et al., 2019), both of them already identified in *Del/Ros1* fruits. Some of the characteristics that anthocyanins conferred to *Del/Ros1* purple tomatoes, such as extended shelf life and reduced susceptibility to postharvest pathogens, were also found in the *Aft/Aft atv/atv* fruits (Bassolino et al., 2013). This indicated that the peel-specific accumulation of anthocyanins, which is predominant in these latter tomatoes, is sufficient to provide them with these important properties.

The attempts to shed light on the regulation of the anthocyanin pathway in tomato started with the same genotypes as those used in breeding programs. For a long time, however, the genes responsible for the *Aft* and *atv* phenotypes were not identified, although, due to the specific inheritance of the two traits (*Aft* being dominant and *atv* recessive), the involvement of an activator and of a repressor of the pathway were hypothesized (Povero et al., 2011). Only in the last three years, also as a result of the greater understanding of the regulation of the anthocyanin biosynthetic pathway, have the natural allelic variations been uncovered that are responsible for the *Aft* and *atv* phenotypes. These studies also confirmed that the combination of specific alleles of these genes is indeed responsible for the complete and uniform purple fruit pigmentation of the *Aft/Aft atv/atv* lines. This thus demonstrated the existence of a strong synergy in the effects of the two

genes, as initially hypothesized on the basis of the analyses of the phenotypes and of the initial molecular data (Povero et al., 2011).

An insertion in the SlMYB-ATV gene underlies the phenotype of the atv plants

The *atv* tomato line, derived from a spontaneous cross between *S. lycopersicum* and *Solanum cheesmaniae* (Rick et al., 1968), shows a general high anthocyanin abundance in the plant, which results in purple leaves, veins, branches, and stems (Fig. 4; Colanero et al., 2018). The phenotype was initially associated with a differential sensitivity to red/far red light, suggesting the involvement of a differential phytochrome-light perception and the classification of the line as a photomorphogenic mutant (Kendrick et al., 1997).

The first genetic mapping of the trait was carried out in the 1960s through the use of genetic markers, which highlighted chromosome 7 as being possibly linked with the phenotype (Rick et al., 1968; Clayberg, 1972). However, this genomic localization has only recently been confirmed. In 2017, through fine-mapping of chromosome 7, Cao et al. (2017) reached a 5-kb resolution of a region linked with the *atv* phenotype. This region harbors the gene *Solyc07g052490* (Table 1) encoding an R3 MYB protein that has been considered as putatively responsible for the trait of study and thus named *SlMYB-ATV*. On the other hand, through genome sequencing of the *atv* line, Colanero et al. (2018) identified the exact location of the region introgressed from *S. cheesmaniae* in chromosome 7 and inside that region identified the same *Solyc07g052490* gene as being responsible for the anthocyanin accumulation.

SlMYB-ATV is mutated and nonfunctional in the *atv* genotype, due to a 4-bp insertion in the second exon of the gene that leads to a frameshift and the onset of a premature stop codon. This produces a short truncated *Slmyb-atv* protein, with loss of the R3 domain (Cao et al., 2017; Colanero et al., 2018). In many plant species R3 MYB proteins act as negative regulators of various processes connected with cell fate determination (Wang and Chen, 2014), including the downregulation of anthocyanin biosynthesis (Zhu et al., 2009; Dubos et al., 2010). SlMYB-ATV is very similar in its structure to other R3 MYB proteins acting as repressors in the anthocyanin biosynthetic pathway, and particularly PhMYBx of petunia (Albert et al., 2014; Colanero et al., 2018).

R3 MYB proteins harbor in their R3 domain the conserved amino acid sequence responsible for the interaction with bHLH proteins (Zimmermann et al., 2004). It is thus believed that R3 MYBs downregulate anthocyanin synthesis by removing bHLH factors from the MBW complexes through competition with the activator R2R3 MYB TFs (Koes et al., 2005; Albert et al., 2014). This was demonstrated for SlMYB-ATV: the mutated *Slmyb-atv* protein cannot bind bHLH factors due to the loss of the R3 domain and thus cannot disrupt the MBW complex (Colanero et al., 2018; Sun et al., 2020). Furthermore, the transcriptional activation of the

SlMYB-ATV gene by the same MBW complex, as previously postulated in the model of the regulation of the anthocyanin pathway (Albert et al., 2014), has been demonstrated (Colanero et al., 2018). Therefore, whereas in wild-type plants SlMYB-ATV acts as a repressor of the MBW complex in a negative feedback loop that fine-tunes anthocyanin production to avoid excessive pigment accumulation, in *atv* plants the lack of a functional R3 MYB repressor is responsible for the misregulated accumulation of anthocyanins wherever their production is induced (Fig. 4).

Aft encodes a functional R2R3 MYB TF that induces anthocyanin pigmentation in tomato fruits

The *Aft* accession derives from an interspecific cross between *S. lycopersicum* and *S. chilense* (Dunal) Reiche (Georgiev, 1972). The fruits of this wild species have purple cloves or spots in their peel, a trait that requires high light activation. This trait in *Aft* becomes a diluted phenotype that consists of spotted pigmentation (Davies et al., 2012) of the skin (Box 1) after exposure to intense light (Fig. 4). Similar to the identification of the *atv* mutation in chromosome 7, the region introgressed from *S. chilense* that contains the genes responsible for the phenotype was identified through genetic mapping (de Jong et al., 2004; Mes et al., 2008; Sapir et al., 2008). This region is located in the distal part of the long arm of chromosome 10, as finally confirmed by two recent studies (Yan et al., 2019; Sun et al., 2020). A genomic region associated with the production of anthocyanins was also mapped on chromosome 10 in eggplant and pepper, showing the existence of a strong synteny for this trait in various domesticated *Solanaceae* (Doganlar et al., 2002; Wang et al., 2018). Also the *Abg* locus, inherited from *S. lycopersicoides*, was mapped on the same chromosome region (Rick et al., 1994); however. it is still not clear if *Abg* is an allele of *Aft*, and it has not as yet been characterized.

Before the final identification of the gene responsible for the phenotype, several R2R3 MYB-encoding genes were identified in the *Aft* introgressed region: *SlAN2*, *SlANT1*, *SlANT1like*, and *SlAN2like* (Table 1; Sapir et al., 2008; Schreiber et al., 2012; Kiferle et al., 2015; Cao et al., 2017; Qiu et al., 2019). They are similar to the orthologous factors involved in anthocyanin regulation in other species (Mathews et al., 2003; Schreiber et al., 2012; Kiferle et al., 2015; Cao, et al., 2017). *SlANT1* and *SlAN2* were considered to be the main genes responsible for the anthocyanin-fruit phenotype (Sapir et al., 2008; Povero et al., 2011; Myers, 2012; Schreiber et al., 2012). In fact, when overexpressed under the constitutive cauliflower mosaic virus 35S promoter, both of these genes can cause a strong anthocyanin pigmentation in all of the main plant organs including the fruits (Kiferle et al., 2015). Therefore, they are functional activators of the pathway. Whereas SlANT1 has not yet been proven to play a physiological role in the plant, SlAN2 has been demonstrated to represent the main R2R3 MYB TF participating in the MBW complex in the

vegetative tissues of tomato (Kiferle et al., 2015). However, later studies revealed very low expression of both genes not only in wild-type fruit peel, but also in *Aft* fruits (Cao et al., 2017; Qiu et al., 2019), thus weakening their possible role in the induction of fruit anthocyanin pigmentation.

Finally, three independent studies were recently published simultaneously, revealing the real genetic basis of the *Aft* trait, consisting of another R2R3 MYB encoding gene, *SlAN2like*, which is closely associated with the previous ones and with *SlANT1like* on the long arm of chromosome 10 (Yan et al., 2019; Colanero et al., 2020; Sun et al., 2020). Interestingly, the identification of *SlAN2like* with *Aft* was obtained through different and complementary approaches. Yan et al. (2019) fine-mapped the *Aft* locus to an interval of ~145-kb on chromosome 10, a strategy that enabled them to exclude *SlAN2*, *SlANT1*, and *SlANT1like* from the candidates. Through CRISPR/Cas9-mediated silencing of *SlAN2like*, they then obtained a much lower accumulation of anthocyanins in the fruits, which was associated with the downregulation of the most important structural genes of the anthocyanin pathway.

Colanero et al. (2020) identified *SlAN2like* as *Aft* primarily through a functional approach. This revealed how, among all the wild-type and *Aft* versions of the four R2R3 MYB TFs encoded by the genes included in the *Aft* introgressed region, only the wild-type version of SlAN2like was nonfunctional. The *Aft* counterpart of the same protein is, on the contrary, fully functional in the activation of the anthocyanin biosynthetic pathway and also expressed at a much higher level in *Aft* fruit peel compared to all the other R2R3 MYB factors. Colanero et al. (2020) identified splicing mutations during the maturation of the precursor mRNA of the wild-type *SlAN2like* gene leading to aberrant mature *SlAN2like* mRNAs. The mutations in the wild-type *SlAN2like* create premature stop codons, which produce short proteins lacking most of the R2R3 domains and all of the C-terminal part of the TF. These proteins were found to be unable to bind the bHLH partners of the MBW anthocyanin complex, thus explaining their inefficacy in the induction of fruit pigmentation.

Sun et al. (2020) focused on the *Aft/Aft atv/atv* 'Indigo Rose' (InR) tomato line. They identified *SlAN2like* through a bulk-population–sequencing approach. Many of the results of the previous articles were confirmed in Sun's study and additional pieces of the puzzle were found. They demonstrated that SlAN2like activates the transcription of both *SlAN1* (Table 1), encoding the bHLH protein that takes part in the key MBW complex of tomato, and of the repressor *SlMYB-ATV*. Sun et al. (2020) also highlighted the competition of SlAN2like and SlMYB-ATV in binding the same bHLH partner SlAN1. They also found the exact location of the splicing mutation in *SlAN2like* that prevents wild-type plants from assembling a correct mature mRNA. Finally, through genetic engineering, Sun et al. (2020) reconstructed all of the network that induces anthocyanin synthesis and accumulation in the fruits of the double mutant *Aft/Aft atv/atv* InR (Fig. 4). They found that: CRISPR/Cas9-mediated silencing of all four R2R3 MYB genes *SlAN2*, *SlANT1*, *SlANT1like*, and *SlAN2like* in InR fruits proved that only *SlAN2like* is necessary to have the anthocyanin-enriched phenotype; and the overexpression of *SlAN2like* in wild-type plants induces a purple anthocyanin pigmentation in the fruit peel. They also revealed that the expression of the wild-type *SlMYB-ATV* gene in InR fruits reverts their phenotype to *Aft*; and, on the other hand, the knock-out of *SlMYB-ATV* in *Aft* fruits increases the pigmentation to that of InR. The essential role of SlAN2like in the induction of the anthocyanin synthesis in the fruits of tomato was thus definitively proved.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Purple tomato fruits are now one of the many variants of color offered on the market to consumers. Because tomato is one of the most consumed vegetables all over the world, anthocyanin-enriched tomatoes can provide our diet with the beneficial amount of antioxidants that preventive medicine recommends. Breeding has mostly exploited the characteristics of *Aft* and *atv* which, combined, produce the maximum amount of pigments in the fruit peel, the tissue that is genetically programmed to synthesize flavonoids. High concentrations of anthocyanins can thus be achieved without negatively impacting other metabolites, enhancing the nutraceutical properties of these tomatoes (Box 2).

OUTSTANDING QUESTIONS

- Given that both *Aft* and *atv* traits derive from wild Solanum species, could other regulators of the anthocyanin pathway be introgressed into tomato from wild species?
- Can the total anthocyanin content be further improved in new tomato varieties produced by conventional breeding?
- Given that anthocyanins accumulate only in the skin of the fruit in purple tomatoes obtained from breeding, how could these pigments also be produced in other tissues of the fruit?
- The uniformity of fruit coloration is key to consumer choice, so is it possible to obtain homogeneous anthocyanin synthesis in tomatoes as a constitutive trait, thus releasing it from environmental triggers?

Recent results have not ended research in this field (see Outstanding Questions). As shown by some genetically engineered purple tomatoes, anthocyanins can also be synthesized in the pulp of the fruit, provided the biosynthetic pathway is appropriately activated. Further studies may thus lead to the identification of new TFs that differentially modulate (either inducing more or repressing less) the pathway in the same peel or in other parts of the fruit. The accumulation of anthocyanins in the flesh would mean that purple tomatoes would not just be consumed fresh but could also be used for processed products, such as pastes, sauces, and juices. The characterization of the *Abg* mutation, stronger than *Aft* in inducing fruit pigmentation, certainly represents an interesting new step in this direction.

The uniformity of pigmentation could also be improved. In fact, the *Aft/Aft atv/atv* purple tomatoes, irrespectively of the background variety where the two alleles were introduced, show a light-dependent and cold-dependent phenotype (Povero et al., 2011). This often restricts the uniform purple coloration, which is so decisive in attracting consumers. Genome editing technologies, which can target the promoter regions of the regulatory genes, could contribute significantly to achieving such uniform coloration.

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