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Special issue: The power of plant specialised metabolism

Review

In pursuit of purple: anthocyanin biosynthesis in fruits of the tomato clade

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Over the past decade, progress has been made in the characterization of anthocyanin synthesis in fruits of plants belonging to the tomato clade. The genomic elements underlying the activation of the process were identified, providing the basis for understanding how the pathway works in these species. In this review we explore the genetic mechanisms that have been characterized to date, and detail the various wild relatives of the tomato, which have been crucial for recovering ancestral traits that were probably lost during evolution from green-purple to yellow and red tomatoes. This knowledge should help developing strategies to further enhance the status of the commercial tomato lines on sale, based on both genome editing and breeding techniques.

Anthocyanins in the tomato: genetically modified organisms (GMOs) and breeding

The first GMO tomato with high levels of anthocyanins in the fruit was reported in 2008 [1]. The recent approval by US government agencies for the marketing of GMO purple tomatoes represents a significative step forward for the nutraceutical enrichment of the human diet[i,ii,iii]. Transgenesis is an important biotechnology to meliorate crops essential for human sustainment. GMOs are often superior to the natural breeding lines thanks to their agronomically relevant traits, often not achievable via conventional approaches. However, EU legislation has so far approved only 18 GMOs in different sectors, and approval is slow and often difficult. Nevertheless, the recent opening of the EU toward the use of genome-editing techniques[iv] [2], as well as the recent growing interest in the rest of the world toward the use of GMOs, highlight how important it is to identify the genes behind the biosynthesis of the nutraceutical compounds in plants and to design genetic strategies to increase these compounds in crops of interest.

Anthocyanins: from plant physiology to human health

Many phytochemicals found in the human diet possess health-promoting properties. Unfortunately, many of these phytochemicals have been excluded from modern diets in favor of more common and unhealthy compounds such as those contained in processed foods. However, the recent growing interest in nutraceuticals has led to a renewed interest in foods based on ancient cereals and their beneficial components.

One such class of compounds are the anthocyanins, which are water-soluble polyphenolic molecules whose colors range from red to purple and blue and which confer typical colorations in the plant kingdom [3,4]. Anthocyanins play a variety of roles in a wide range of plant species, tissues, habitats, and environmental conditions [4,5]. In the subepidermal cell layers of the vegetative tissues, anthocyanins act as a photoprotective screen by absorbing excess light, including UV-B radiation, which is harmful for the photosynthetic apparatus; this protective role is evidenced by the timing of induction of the pathway, which is fast under high light and/or low temperature conditions when the dark reactions of photosynthesis cannot keep pace with the

Highlights

Anthocyanins are natural compounds that act as antioxidants and pigments and are produced by plants in response to stress and various environmental conditions.

Anthocyanin-rich diets protect against many chronic diseases, a wide range of tumors, and inflammatory diseases.

The common tomato (*Solanum lycopersicum*) can synthetize anthocyanins only in the green parts of the plant, but not in the fruit. Recent studies have shown that through genetic engineering and breeding it is possible to re-establish the synthesis of anthocyanins in the fruit.

The first purple genetically modified tomatoes were first approved for sale in the USA in 2022.

Tomato breeding has led to the development of non-genetically modified (GMO) purple tomatoes by exploiting wild relatives of *S. lycopersicum*. They contain anthocyanins in the fruit epicarp and have a higher antioxidant power than red tomatoes. They represent a novel nutraceutical food and can be sold in the EU.

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light reactions, leading to photo-oxidative stress [6–8]. Other stress conditions, both biotic and abiotic, cause plants to produce more reactive oxygen species (ROS), and this often results in the accumulation of anthocyanins which help to protect cells, even after cellular damage, through scavenging and metal-chelating activities [9–11]. Anthocyanins also color flowers and fruits of many species to attract pollinators and seed-dispersing animals [6]; they also prolong the shelf-life of tomato fruits by slowing the process of over-ripening and reducing the susceptibility to necrotrophic pathogens such as *Botrytis cinerea* [12,13].

There are many different anthocyanin molecules, and different anthocyanin classes (Box 1) appear to offer different physiological and ecological functions based on their structure and antioxidant power [14–16].

Box 1. Anthocyanins: insights into their molecular structure and biosynthesis

Anthocyanins are a class of water-soluble polyphenolic compounds which belong to the flavonoid family of specialized plant metabolites [3]. They are glycosylated forms of anthocyanidins, the molecular structure of which involves two aromatic rings (A and B), a core heterocyclic oxygen ring (C), and a C3 bridge, referred to as a C6–C3–C6 structure (Figure IA). Because of the structural complexity of these molecules, which shows different patterns of hydroxylation, methoxylation, and acylation, the members of this family have increased in number since their first identification, and more than 700 different compounds have been reported in the plant kingdom [16,22,38,82,83].

Anthocyanin biosynthesis is conserved and well characterized in higher plants [5] (Figure IB). Anthocyanins derive from the phenylpropanoid pathway (PPP), which first produces phenylalanine which is converted to cinnamic acid and then coumaroyl-coenzyme A (CoA) by the consecutive activities of the enzymes phenylalanine ammonia lyase (PAL), cinnamate-4-hydroxylase (C4H), and 4-coumaryl-CoA ligase (4CL). Subsequently, several structural genes encode enzymes in the pathway committed to anthocyanin biosynthesis, which is traditionally divided between the early biosynthetic genes (EBGs) and the late biosynthetic genes (LBGs), encoding the enzymes involved in the synthesis of the common precursors of other flavonoids and of the anthocyanidins, respectively (Figure IB). These enzymes have been suggested to be organized into a metabolon that is associated with the cytoplasmic face of the endoplasmic reticulum [5,84–86] (Figure IB). Chalcone synthase (CHS) activity allows the synthesis of naringenin chalcone by combining three molecules of malonyl-Co-A with one molecule of coumaroyl-Co-A [17,84]. Chalcone isomerase (CHI) isomerizes naringenin chalcone to naringenin. Naringenin is then converted into dihydrokaempferol by flavanone 3-hydroxylase (F3H), which can be further hydroxylated by flavanone 3′-hydroxylase (F3′H) or flavanone 3′,5′-hydroxylase (F3′5′H) to produce dihydroquercetin or dihydromyricetin, respectively. The dihydroflavonols are substrates of dihydroflavonol 4-reductase (DFR), whose activity leads to the synthesis of the colorless leucoanthocyanidins, which are converted into colorful anthocyanidins by anthocyanidin synthase (ANS). Flavonoid 3-O-glucosyltransferase (UFGT) and other glycosyltransferases add sugar moieties (principally glucose, arabinose, rhamnose, and galactose) to the C3 position of the anthocyanidin to form anthocyanins, and these may be further methylated by specific methyltransferases and acylated with aromatic or aliphatic acyl groups by acyltransferase (AAT) [57,86–88]. In the tomato there is a single aromatic acyl transferase that adds a coumaroyl group to the C4 position of the rhamnosyl moiety of the anthocyanidin rutinoside. Interestingly, expression of the genes encoding the methyl and acyl transferases and the anthocyanin transporters are regulated by the MBW complex [1].

Following their biosynthesis, anthocyanins are transported into the vacuole to be stored or further modified by vacuole-localized enzymes [89] (Figure IB). Two main transport mechanisms have been described, which may act independently, together, or in a mutually exclusive manner: direct transport to the vacuole, thanks to tonoplast-localized factors, represented by multidrug and toxic compound extrusion (MATE) and ATP-binding cassette (ABC) transporters, whose action is mediated by glutathione S-transferases (GST), or vesicle transfer from the secretory pathway [90–95].

Anthocyanins can be classified into three main classes based on their B-ring hydroxylation pattern; the basic representatives are monohydroxylated pelargonidin, dihydroxylated cyanidin, and trihydroxylated delphinidin [17,23] (Figure IB). Peonidin, petunidin, and malvidin are produced by further methylation reactions [17]. The color of an anthocyanin is primarily influenced by the pattern of hydroxylation on the B ring of the core structure [17]. pH can further affect the molecular structure of anthocyanins, resulting in different chemical species characterized by different colors [17], and also their stability at levels above pH 7, possibly leading to degradation depending on the substituent groups [23]. The main anthocyanins detected in SunBlack [78] and Indigo Rose [96] varieties were petanin [petunidin-3-(p-coumaroyl)-rutinoside-5-glucoside] and negretein [malvidin-3-(p-coumaroyl)-rutinoside-5-glucoside], while petanin and nasunin [delphinidin-3-(p-coumaroyl)-rutinoside-5-glucoside] were detected as main anthocyanins in the *Del/Ros1* line [88]. The differences in anthocyanin species between the GM tomatoes and the introgression lines are likely to be due to differential activities of decorating enzymes in the different genetic backgrounds.

Glossary

Alternative splicing (AS): the process which – starting from a primary transcript and through different arrangements of exons (i.e., exon skipping and intron retention) – leads to different alternative mature mRNAs. These can be translated into protein variants with different activities or functions or can represent aberrant transcripts that are finally degraded by nonsense-mediated decay mechanisms.
Basic helix–loop–helix (bHLH): a transcription factor belonging to one of the largest families in plants; bHLHs regulate growth and developmental processes and stress responses.
Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9): a widely used method to perform gene editing in eukaryotic cells through RNA-guided nucleases derived from the microbial adaptive immune system. It can be used to edit genes by cutting DNA precisely in target positions followed by DNA repair that generates mutations at the repair site by non-homologous end-joining (NHEJ).
miRNAs: small RNAs that act as negative regulators of their targets by promoting mRNA degradation through the specific cellular machinery, or by inhibiting mRNA translation by preventing the binding of the ribosome to the transcript.
MYB–bHLH–WDR (MBW) complex: a multiprotein complex regulating anthocyanin synthesis; it is made up of proteins belonging to the R2R3-MYB, bHLH, and WDR families. Within the complex, R2R3-MYB factors confer selectivity on target genes, leading to the accumulation of anthocyanins in specific tissues or at certain times. bHLH factors increase the specificity of the transcriptional activation, likely offering additional regulatory mechanisms to ensure specific recognition of the DNA binding sites, including binding to specific binding motifs in at least some of their target gene promoters and/or activation of the transcriptional process itself. The WDR proteins are believed to have the least specific role, and their levels show a sort of constitutive behavior. Nevertheless, their presence is essential for activating the anthocyanin pathway (e.g., in tomato and other solanaceous plants), and knockout WDR proteins or mutations that alter

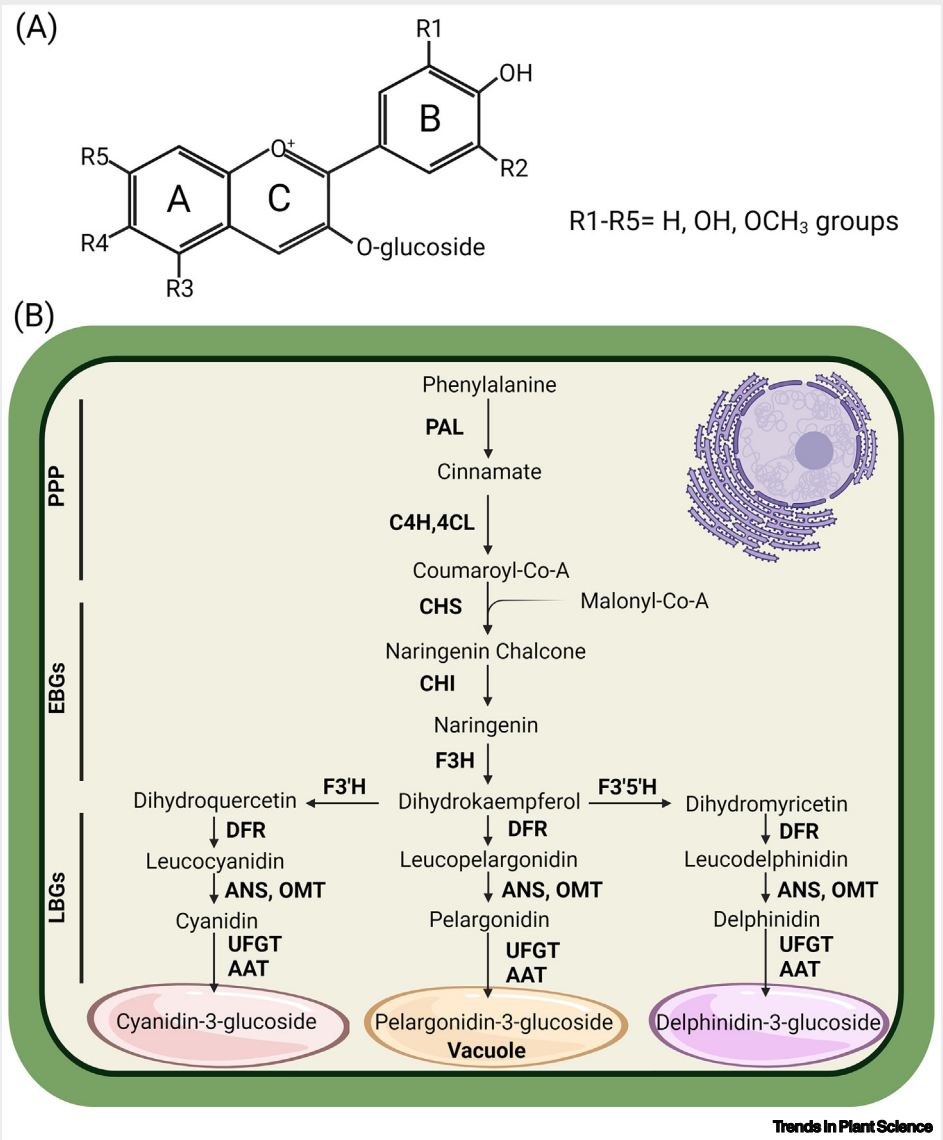


Figure I. Anthocyanin structure (A) and anthocyanin biosynthesis (B). Abbreviations: AAT, acyltransferase; ANS, anthocyanidin synthase; C4H, cinnamate-4-hydroxylase; CHI, chalcone isomerase; CHS, chalcone synthase; 4CL, 4-coumaryl-coenzyme A ligase; DFR, dihydroflavonol 4-reductase; F3H, flavanone 3-hydroxylase; F3′H, flavanone 3′-hydroxylase; F3′5′H, flavanone 3′,5′-hydroxylase; OMT, O-methyltransferase ; PAL, phenylalanine ammonia lyase; UFGT, flavonoid 3-O-glucosyltransferase.

their stability lead to completely anthocyanin-less plants.

Myeloblastosis (MYB) factor: a transcription factor belonging to a family of transcription factors among the largest in plants; they act in several processes, including plant and cell growth and development, regulation of primary and secondary metabolism, and stress responses. MYB proteins comprise a conserved MYB DNA-binding domain made up of one to four imperfect repeats, each with a combined length of roughly 50 amino acids, plus a transactivation domain.

R2R3-MYB: transcription factors of the MYB family that guide the activation or repression activity of the MBW complex binding to the promoters of the structural genes. R2R3-MYB factors share in the R3 repeat the conserved domain of interaction with bHLH factors characterized by the motif (D/E)Lx2(R/K)x3Lx6Lx3R. The repressors can exert their activity by subtracting bHLH factors from the R2R3-MYB activators and/or thanks to specific functional motifs in their C-terminal domains which may interact directly with promoters of biosynthetic genes.

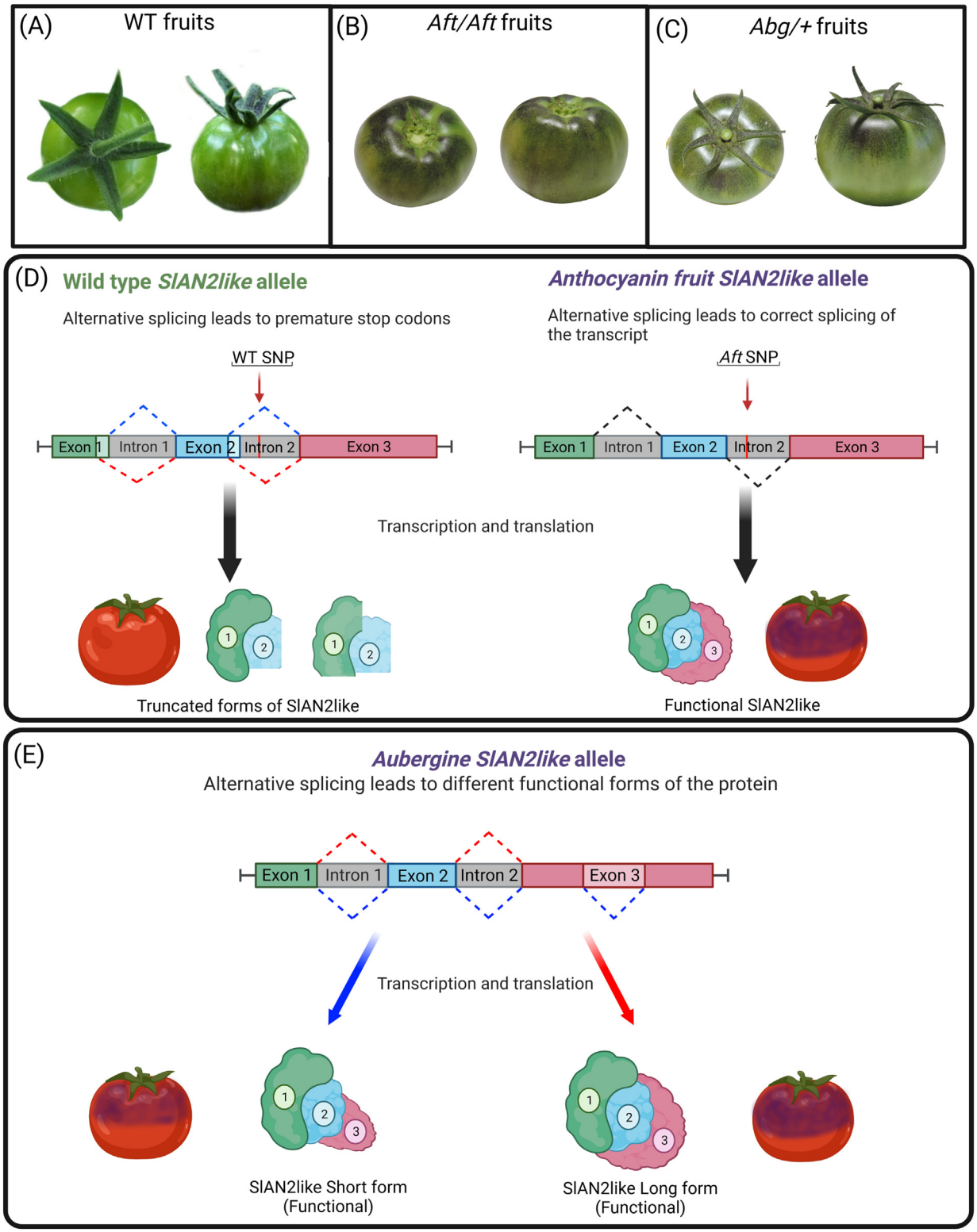
R3-MYB: a repressor of the anthocyanin biosynthetic pathway which shows the conserved domain of interaction with bHLH factors in the R3 repeat. This motif allows them to act as passive inhibitors, by subtracting bHLH factors from the MYB activators.

Single-nucleotide polymorphisms (SNPs): genetic variations at single base positions between different genomic sequences.

WDR: proteins that contain the WD40 repeat, a short structural motif of approximately 40 amino acids, often terminating in a tryptophan (W)–aspartic acid (D) dipeptide. They are important components of multiprotein complexes such as the MBW complex, but their exact role and presence in all the MBW complexes is often debated.

As proved by the vibrant hues of many vegetables and fruits, anthocyanins and other plant antioxidants (e.g., carotenoids) are already part of the diet of many animals, including humans. Research into novel functional foods has explored new means of crop development, and biotechnological techniques have played a substantial role [17]. The tomato (*Solanum lycopersicum*) was an excellent candidate for this exploration because its fruits do not produce anthocyanins.

The development of the purple GMO tomato line by Butelli *et al.* in 2008 [1] was followed by the demonstration, on this specific line, of the health properties of anthocyanins in these fruits,



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characterized by an incredibly high antioxidant content. The health effects of these tomatoes were demonstrated *in vivo* on P53 knock-out mice, significantly extending the lifespan of the mice when they were fed with the tomatoes [1].

Over the years, more anthocyanin-pigmented tomato varieties have been marketed [18]. Anthocyanin enrichment in the fruit flesh has been achieved only in GMO lines, whereas varieties based on natural breeding show accumulation of anthocyanins only in the fruit peel [19]. All current preclinical studies demonstrating health benefits have been performed with GMO varieties, but purple fruits from the varieties obtained by conventional breeding might exert similar health effects, although, because anthocyanins are synthetized only in the peel, a considerably greater consumption would be required to reach the same intake levels. Any benefits would also be lost if the peel were removed during the fruit processing, a reason for further improvement of the traits with the target of producing tomatoes with purple flesh.

Despite their low bioavailability, dietary anthocyanins have been associated with a variety of health benefits, traditionally ascribed to their antioxidant activities [20–23]. They have anti-atherosclerotic, anti-diabetic, and antitumor actions. They can also reduce the risk of cardiovascular diseases, manage body fat accumulation and diabetes, improve visual and cognitive functions, and provide neuroprotective effects [24–28]. The modulation of many signaling pathways may contribute to these outcomes [29,30]. For example, cyanidin appears to alleviate inflammation by inhibiting the signaling of the proinflammatory cytokine interleukin-17A [31]. Anthocyanins may exert their function by also influencing the composition and functioning of the gut microbiota [32–36], and they positively impact the abundance of several beneficial species – such as those belonging to the Peptostreptococcaceae, *Bifidobacterium, Lactobacillus,* Actinobacteria*, Prevotella,* Bacteroidetes*, Akkermansia,* Ruminococcaceae, and *Alloprevotella* – as well as reducing the abundance of species correlated with negative health outcomes [32,37]. Especially in conditions such as obesity and diabetes, anthocyanins showed other prebiotic effects on the gut, such as decreased triglycerides, total cholesterol, steatosis scores and insulin resistance index, and ability to inhibit harmful bacteria [37]. However, the molecular mechanisms underlying these health benefits are still largely unknown and the object of active research.

The road to purple tomatoes: attempts and achievements

Fruits of *S. lycopersicum* are among the most consumed vegetables worldwide. Wild-type (WT) tomato varieties do not synthetize anthocyanins in fruits (Figure 1A); nevertheless, they contain all the biosynthetic genes required to do so [18,38,39]. By contrast, tomatoes contain large amounts of carotenoids (with lycopene as the most abundant compound), sugars, amino acids, and vitamins, as well as some flavonols and other phenylpropanoids. They also contain many volatile molecules, which confer their intense flavor. This high nutritional value has made the tomato a good candidate for anthocyanin enrichment, to further boost its nutraceutical properties [18].

Early studies investigating the lack of synthesis of anthocyanins in tomatoes focused on expression of the *CHI* gene (Box 1). When *CHI* was ectopically expressed in the fruit, it conferred a 78-fold

Figure 1. AN2like confers the pigmentation of *Anthocyanin fruit (Aft)* and *Aubergine* (*Abg*) fruits. Fruits at the mature green stage of wild-type (WT) (A), *Aft* homozygous (B), and *Abg* heterozygous (C) genotypes in the domesticated tomato genetic background. (D) WT and *Aft SlAN2like* allelic structures with splice sites, corresponding proteins from the mature mRNAs, and relative phenotypes. The position of the SNP responsible for generating the alternative splice site is highlighted in red. (E) *SlAN2like* allelic structure in *Abg* with the different splicing sites leading to two different proteins from the relative mature mRNAs and relative phenotypes. The numbers on the protein structures represent the polypeptides derived from the corresponding exons. Presumably, in the short form of *SlAN2like,* two alternative splice sites within the third exon allow the production of an alternative transcript of *SlAN2like,* which splice out a small inner sequence located in this region. The resulting short transcript still retains the first and the second exon but only the initial and the last part of the third, which is, however, still in frame until the canonical stop codon.

increase in flavonol content, but no anthocyanins were synthesized, suggesting that something else was missing or blocking the pathway [39,40].

When the central role of the regulatory **MYB–bHLH–WDR (MBW) complex** (see Glossary) in anthocyanin synthesis started to become clear (Box 2), various studies tested the heterologous expression of transcription factors (TFs) of the complexes already identified in other species, such as the **R2R3-MYB** factor C1 with the **basic helix–loop–helix (bHLH)** factor Lc from *Zea mays* or the *Arabidopsis thaliana* R2R3-MYB factor PAP1. These tests either failed or were only partially successful, with no or only a small increase in the anthocyanin content [41,42].

As already reported, the most successful attempt was carried out in 2008 [1] when two TFs from *Antirrhinum majus* (snapdragon), the bHLH factor *Delila* (*Del*), and the R2R3-MYB *Rosea1* (*Ros1*), were expressed under the control of the *E8* promoter, specific to tomato fruit. The transgenic lines obtained produced the first deep purple tomato, with a uniform coloration from the peel to the flesh. The health properties demonstrated led first to the US Department of Agriculture/Animal

Box 2. Anthocyanin synthesis: a highly regulated process

The anthocyanin biosynthetic pathway responds to multiple environmental and developmental signals. It is tightly regulated at the transcriptional, post-transcriptional, and post-translational levels, as shown in Figure I. The primary level of regulation is represented by the expression of the regulatory genes, which is affected by the different upstream signal transduction mechanisms [76,97,98], and allows the production of the TFs, which then control the expression of the structural genes coding for the enzymes of the pathway [18]. The MBW complex, made up of R2R3-MYB, bHLH, and WDR proteins, is highly conserved in plants and regulates the activity of key biosynthetic genes of the pathway [97]. MYB TFs can be activators or repressors of the anthocyanin synthesis [76]. In the tomato, a special anthocyanin locus lies in the long arm of chromosome 10, which contains six *R2R3-MYB* genes, *SlANT1*, *SlAN2*, *SlANT1like, SlAN2like, SlAN2like-2* (also known as *SlMYB113),* and *SlMYB32*, which all code for TFs involved in the pathway [50,58,77]. The first four are activators, while SlMYB32 (also known as SlTHM27) is a repressor [58,77]. SlAN2like-2 is not functional in *S. lycopersicum*. There is a high level of synteny for this locus, as it is also present in other members of the Solanaceae, such as eggplant, potato, and pepper [99,100]. There appear to be hierarchical systems that control the role of bHLH factors [76,101]. In the Solanaceae, for example, two different complexes have been identified: the first one, formed by a R2R3-MYB (e.g., SlAN2 in tomato vegetative tissues, or SlAN2like in tomato fruit) and the bHLH factor SlJAF13, activates the expression of the gene *SlAN1*, encoding the second bHLH [101]. SlAN1 participates in the second complex with the R2R3-MYB proteins, which activates the expression of genes encoding enzymes in the pathway [52,101]**.** SlJAF13 and SlAN1 are so far the only known bHLHs taking part in the Solanaceae MBW complex, and mutations in these genes can result in an anthocyanin-less phenotype. *SlAN1* [48], for example, was recently identified as the gene mutated in plants exhibiting the well-known *Hoffman's anthocyanin-less* phenotype, causing loss of anthocyanin synthesis in the tomato, a gene controlled by developmental and environmental factors, and strongly induced by cold [102]. *SlAN1* is the homologue of Petunia *PhAN1,* controlling anthocyanin accumulation in petals, anthers, and leaves [103]. In *Solanum tuberosum,* AN1 and JAF13 are similar but different, in fact *PhJAF13* expression fails to activate anthocyanin synthesis when expressed in *Nicotiana benthamiana*, while potato *AN1* can [104]. WDR proteins have often been discussed for their actual role in regulating anthocyanins, taking part also in the MBW complexes. One WDR encoding gene is known in tomato, *SlAN11* [48]. The exact role of this factor is still not completely known, but experimental evidence has shown how it interacts with SlAN1 to promote anthocyanin regulation [105]. Apparently, SlAN11 does not interact directly with the R2R3-MYB factor, but only with the bHLH in the MBW complex [105]. It is known that silencing *SlAN11* results in lower anthocyanin activation, while its expression levels in WT plants appear to be constitutive, suggesting that the WDR protein is probably essential but not sufficient for the anthocyanin activation [105].

At the post-transcriptional level various mechanisms have been described, the most common being **miRNA** regulation and alternative splicing (AS), as shown in Figure I. The most evolutionarily conserved and studied miRNA in plants is miR156. In several species miR156 negatively targets *SPLs* gene transcripts which, apart from their well-known role in development, also affect anthocyanin synthesis [106–108]. Other miRNAs, such as miR828 and miR858, target different *MYB* genes involved in positive or negative regulation of the flavonoid pathway [109,110]. Anthocyanin synthesis is regulated through AS [111,112] in different species. In fact, many genes in the flavonoid pathway, both structural and regulatory, exhibit AS [113,114]. Among them, the biosynthetic gene *DFR* in some species of the spiny *Solanum* group [115], and the regulatory gene *SlAN2like*, encoding the R2R3-MYB factor responsible for the anthocyanin pigmentation of the tomato fruit exocarp [53,54]. Finally, a well-known mechanism of post-translational regulation is the substrate specificity of the enzyme DFR (see Box 1 in the main text). In tomato, for example, the predominant biosynthetic pathway leads to the synthesis of delphinidin, which confers a typical dark purple color, due to the substrate selectivity of DFR which shows a preference for dihydromyricetin, the precursor of delphinidin, over the other dihydroflavonols [116].

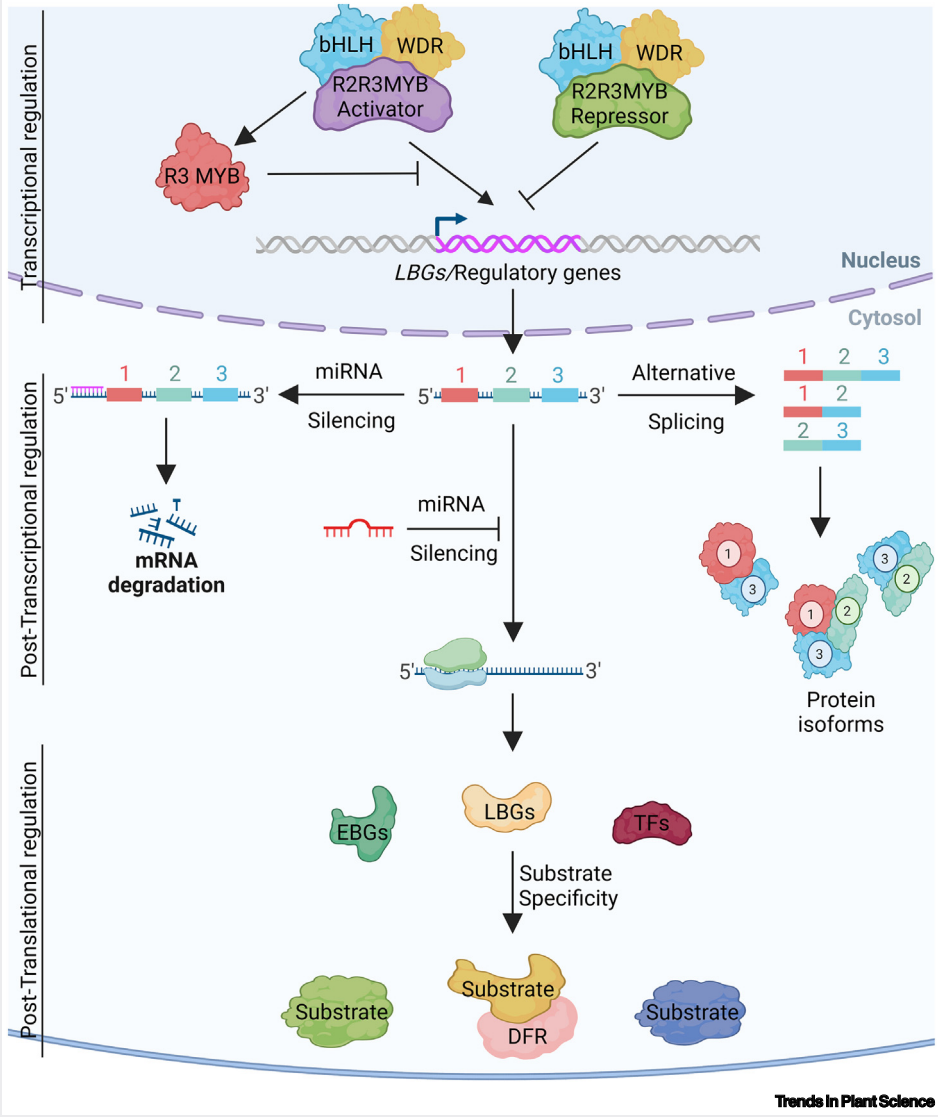

Figure I. Regulation of anthocyanin biosynthesis. Abbreviations: bHLH, basic helix–loop–helix; DFR, dihydroflavonol 4-reductase; EBG, early biosynthetic gene; LBG, late biosynthetic gene; TFs, transcription factors; WDR, proteins that contain the WD40 repeat.

and Plant Health Inspection Service (USDA/APHIS) approval in September 2022 and later to FDA notification in July 2023. Eventually, the GMO purple tomatoes arrived on the market in the USA where they are having considerable commercial success[i,ii,iii].

EU laws have been restrictive towards cultivation and commercialization of GMO food, and so far only 18 GMOs have been approved for different uses, including food, feed, or cultivation. Purple tomatoes have not yet been submitted for approval in Europe.

Targeted breeding represents an alternative strategy to improve performance and quality traits, and exploitation of natural biodiversity within the broader tomato family has allowed the

production of tomato varieties with fruits enriched in anthocyanins. Indeed, breeders and researchers noticed that, unlike the cultivated species, some wild relatives of *S. lycopersicum* showed a dark purple pigmented fruit phenotype [43]. These different loci were then introduced through guided breeding into the tomato genome to trigger anthocyanin synthesis in tomato fruits. The best-known loci which effectively boosted the production of anthocyanins in the fruit peel were *Anthocyanin fruit (Aft)*, *atroviolacea (atv)*, and *Aubergine (Abg)* [43].

Aft allows the synthesis of anthocyanins in tomato fruits by re-establishing the key R2R3-MYB factor

Of all the tomato traits exploited by breeders, *Aft* was the most effective. The *Aft* locus from *Solanum chilense* [44], a wild relative in the genus *Solanum*, promotes the synthesis of anthocyanins in the fruit peel of tomato, a dominant phenotype which depends on exposure to strong light or low temperatures, and which leads to fruits with a purple-spotted skin [43–45] (Figure 1B). *Aft* was first reported by Georgiev *et al.* [44] in an introgression from *S. chilense*. Jones *et al.* [46] hypothesized that *Aft* might be an allele of *Abg*, a putative regulatory gene inducing anthocyanin pigmentation in tomato fruit peel and already mapped on chromosome 10. Sapir *et al.* [47] attempted to extend the knowledge behind this trait and again reported the possible localization of *Aft* on chromosome 10. Located on the same chromosome [48], the R2R3-MYB genes *SlAN2* and *SlANT1* were initially thought to be responsible for the *Aft* phenotype [49,50], but Yan *et al.* [51] then fine-mapped the *Aft* locus and identified a single gene, *SlAN2like (Solyc10g086290),* encoding a TF belonging to the R2R3-MYB family and very similar to the proteins encoded by other nearby genes *SlANT1, SlAN2,* and *SlANT1like* (Box 2). Very few mutations were found in the conserved domains of *SlAN2like* in comparison to WT and *Aft* alleles, with no predicted impact on the final proteins. However, **Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9)-**mediated knock-out of *SlAN2like* in an *Aft* genotype resulted in anthocyanin-free fruits with a strong downregulation of all biosynthetic and regulatory genes. Yan *et al.* [51] demonstrated the role of *SlAN2like* as a gene regulating anthocyanin biosynthesis and suggested that the differences between *Aft* and WT plants may be due to alterations in the expression of *SlAN2like*.

Colanero *et al.* [52] and Sun *et al.* [53] showed that *SlAN2like* regulates anthocyanin biosynthesis in fruit peel, as its levels in *Aft* are much higher than those of the other genes encoding R2R3-MYB factors expressed there, and they then found a difference between *Aft* and WT alleles of *SlAN2like*. Functional characterization of *SlAN2like* showed that, unlike the *Aft* allele, the WT allele carries a mutation affecting mRNA splicing (Box 2) which prevents the correct expression of the TF. In fact, some key **single-nucleotide polymorphisms (SNPs)** in the WT *SlAN2like* allele generate **alternative splicing (AS)** sites, which are given preference over the canonical sites and lead to the production of aberrant transcripts. These transcripts are later translated into truncated proteins which lack the C-terminal activation domain and most of the R2–R3 domains of the TF. They are thus unable to interact with the other components of the MBW complex, bHLH and **WDR** factors (Figure 1D). This explains the inability of the WT allele to activate the synthesis of anthocyanins in the fruit peel. Indeed, if a correctly spliced coding sequence is reconstructed from the WT *SlAN2like* allele and overexpressed in tomato protoplasts, it can activate the expression of the key biosynthetic gene *DFR* (Box 1).

Based on these data, it is possible to establish a model for anthocyanin synthesis in tomatoes. SlAN2like, acting as the most expressed anthocyanin R2R3-MYB activator in the fruit peel, can recruit the first bHLH factor JAF13 and, likely together with the AN11 WDR protein, activate the expression of the bHLH *AN1* gene. The second MBW complex thus formed, together with AN2like, AN1, and AN11 proteins, may activate the late biosynthetic genes (LBGs) required for anthocyanin synthesis [52].

Aubergine unveiled: an allele of *SlAN2like* with a novel mechanism of regulation

The tomato *Abg* accession was obtained from interspecific crosses between *S. lycopersicum* and *Solanum lycopersicoides*, another wild relative [54]. The phenotype of *Abg* is similar, but not identical, to *Aft*; *Abg* confers the ability to synthesize anthocyanins in the fruit epicarp upon exposure to high light levels in *S. lycopersicoides* or when introgressed into *S. lycopersicum* (Figure 1C); however, the degree of anthocyanin accumulation can range from purple-spotted fruits to intense homogeneous jet-black colorations [43].

Rick *et al.* [54] first mapped *Abg* to chromosome 10, on the same long arm as *Aft* in *S. chilense* [52], and where Pertuzé *et al*. [55] further described a paracentric inversion. The *Abg* line drew interest from breeders because its fruit phenotype is the most intensely pigmentated of tomato anthocyanin mutants, which led to the name '*Aubergine'* due to its similar coloration to eggplant. Unfortunately, the *Abg* locus is unstable, and the homozygotes are sterile. Thus, there were no further breeding or allele tests with *Aft* [43].

More recently, Powell *et al.* [56] sequenced the genome of the wild relative of tomato, *S. lycopersicoides*, and showed that this species predominantly expressed *SlAN2like* in the fruit. The genomic sequence of *S. lycopersicoides* showed the same SNP in the 5′ splice site of the second intron of *SlAN2like* as in the *Aft* allele, where it was shown to be essential for the correct splicing of the transcript [57].

Menconi *et al*. [58] defined the molecular and genetic basis of *Abg* in the introgressed *S. lycopersicum* line showing for *AN2like,* the same SNP in the 5′ splice site of the second intron as in *Aft,* required for the correct maturation of the transcript, and confirming that *SlAN2like* in *Abg* is correctly spliced (Figure 1E). It can therefore activate the expression of its target genes, and its expression in fruits leads to strong pigmentation [58]. *SlAN2like* silencing in *Abg* fruits completely abolishes anthocyanin synthesis in the epicarp, proving the role of the gene in the phenotype [58].

Interestingly, a second transcript of *SlAN2like* was described in this genotype as having resulted from AS, leading to a shorter mRNA than the canonically spliced one [58] (Figure 1E). This short form of *SlAN2like* encodes a protein less efficient at transcriptional activation due to the lack of a region in the C-terminal domain containing part of a functional motif known as S6B [59]. The short *SlAN2like* transcript was much less expressed than the longer one, perhaps representing a form of regulation operating in response to developmental or environmental stimuli.

During the resequencing of *S. lycopersicoides,* Powell *et al.* [56] also identified an additional *R2R3-MYB* gene on chromosome 10, which they named *SlydAN2like-2* due its similarity to *SlAN2like*. This gene was shown to have been introgressed into the *Abg* locus [58], and according to sequence similarities, phylogenetic analysis, and the presence of motifs typical of anthocyanin activators already named as MYB113, was also called *MYB113*. This gene is expressed in the vegetative parts of the plant, but transcript levels are negligible in the fruit [58]. Interestingly, this gene has been inactivated following multiple mutations in the domesticated tomato, as well as in all the *Solanum* wild relatives close to *S. lycopersicum* [56,58].

Purple fruits in *Solanum galapagense*: different origin but same mechanism – another allele of *SlAN2like*

Tomato wild relatives can be classified in two major phylogenetic groups: the green-fruited clade, and the red- and yellow-fruited clade [60,61]. The first group includes the most phylogenetically distant species, while the second contains the species closer to the cultivated *S. lycopersicum* varieties. Green-fruited tomatoes often show purple-colored fruits, with anthocyanin stripes or

spots, while yellow and red fruits do not. This may mean that the ability to synthesize anthocyanins in the fruit peel was lost during evolution from green- to red-fruited tomatoes, such as those produced by the ubiquitous *S. lycopersicum* varieties and by *Solanum pimpinellifolium*, considered its immediate ancestor species. The anthocyanin-pigmented phenotype may have been lost as the *Solanum* species migrated towards lower altitudes or were counter-selected by humans during domestication due to the more appealing red phenotype of the fruits.

Interestingly, *S. galapagense*, an endemic species of tomato from the Galápagos Islands – which belongs to the red and yellow clade and is thus very close to *S. lycopersicum* – shows a dark purple fruit pigmentation in the accession LA1141 [62]. *S. galapagense* is not only different from other wild species due to its yellow fruit, but also due to its yellow or green leaves, drought resistance, small seeds, and long seed dormancy [63]. Furthermore, this species is highly compatible for crossing with cultivated tomato, and is thus an easy, accessible source of genetic diversity. This has already led to interesting novel alleles, including *jointless pedicel* (j^2), *arthritic articulation* (j^{2in}) which allow mechanical harvest, and *anthocyanin gainer* (ag^2) and *Beta (B)*, which confer high β-carotene content [63–67].

Fenstemaker *et al.* [62] recently tested the origin of *S. galapagense* from the hybridization of *S. pimpinellifolium* and *Solanum habrochaites* and revealed the basis of the anthocyanin synthesis in the fruits of the line LA1141. Two different loci responsible for fruit pigmentation were identified, one conferring the synthesis of anthocyanins in the fruit, and producing, when present, populations that had light purple fruits, and a second one that, when combined with the first, generated populations with fruits with deep purple skins (Figure 3B). The results showed that the first essential mechanism for anthocyanin synthesis in this line was the same as that shown by the green-fruited clade species, that is, the presence of a functional allele of the gene *SlAN2like*, as observed first in *Aft* and in *Abg* and *S. lycopersicoides* [51–53,56,58]. Interestingly, the phylogenetic data showed that this allele did not derive from an introgression from the green-fruited clade, but rather from a gain-of-function mutation that evolved from a nonfunctional *SlAN2like* allele backward, thereby restoring its function. The second locus, which, when combined with the first one, generated deep purple fruits in the line LA1141, was identified on chromosome 7 as a nonfunctional allele of the repressor *SlMYB-ATV* gene [62]. Knockout of *SlMYB-ATV* increases anthocyanin content in the foliage and in the fruit peel when combined with a functional allele of *SlAN2like* [51,53,68].

Anthocyanin synthesis can be boosted by knockout of the *atv* gene

An interesting result obtained through breeding was the use of the tomato line *atv*. This phenotype was introgressed into *S. lycopersicum* from the wild relative *Solanum cheesmaniae*, another species from the Galápagos Islands. The *atv* phenotype was first described by Rick *et al.* [69] as having a generally high content of anthocyanins in all the vegetative parts of the plant, often resulting in dark purple veins, stems, and leaves (Figure 2B), as well as an altered perception of red and far-red light, which suggested an impairment in the mechanism of light perception [70].

The *atv* line rapidly became popular with breeders not only for its phenotype, but also for its synergy when combined with other genotypes harboring the *Aft* trait [51,68,71,72].

The *atv* trait was mapped to chromosome 7 [69], and the *atv* locus was identified by Cao *et al.* [71] through fine mapping in a small region on chromosome 7, which encodes for only one protein belonging to the **myeloblastosis (MYB) factor** family, and thus named SlMYB-ATV. This protein showed high phylogenetic similarity with putative homologs in *A. thaliana* and *Petunia hybrida* –

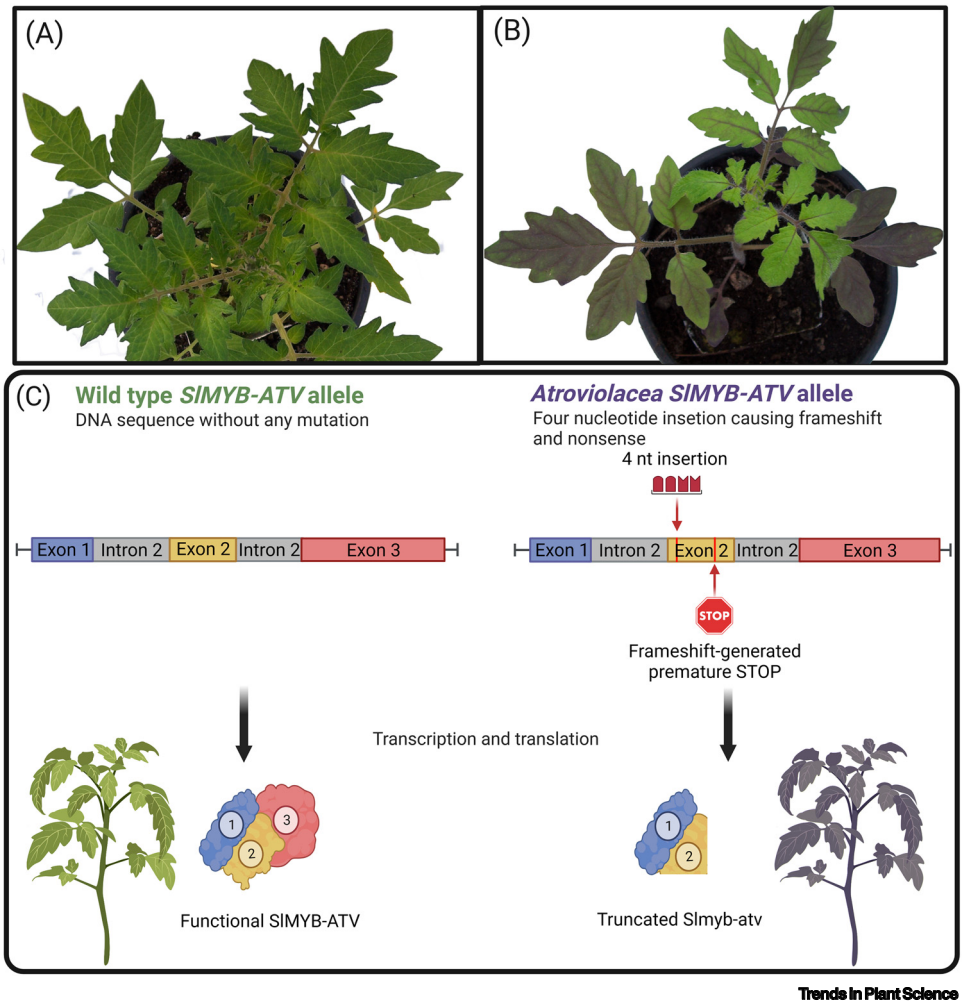

Figure 2. Loss of function of SlMYB-ATV boosts anthocyanin synthesis. Phenotype of young wild-type (WT) plants (A) and *atroviolacea (atv)* plants accumulating high levels of anthocyanins in leaves, stems, and leaf veins (B). (C) *SlMYB-ATV* allelic structure in WT plants and in *atv* plants. In the *atv* allele a 4-nt insertion results in a premature stop codon at the end of the second exon, leading to a truncated and nonfunctional protein. The numbers on the protein structures represent the polypeptides derived from the corresponding exons.

AtCPC and PhMYBx, respectively – shown to act as anthocyanin repressors. Loss-of-function mutations in such genes result in plants with higher anthocyanin content [73].

Cao *et al*. [71] showed that enhanced anthocyanin content was correlated with enhanced expression of biosynthetic genes in *atv* plants expressing a truncated form of SlMYB-ATV. In fact, in *atv* plants *SlMYB-ATV* harbors a 4-bp insertion in its coding sequence which is predicted to cause a premature stop codon and a truncated SlMYB-ATV factor (Figure 2C). The data supported the hypothesis of *SlMYB-ATV* as a candidate gene for the *atv* locus and also, due to the recessive nature of its phenotype, as a repressor of anthocyanin production. Colanero *et al.* [72], confirmed this using genome sequencing coupled with functional characterization of the *ATV* gene.

Phylogenetic analysis showed that SlMYB-ATV belongs to the CPC-like subgroup of the **R3-MYB** family, a well-known group that includes many anthocyanin repressors [73–76] that exert

their activity by competing with the R2R3-MYB transcriptional activators for interaction with the bHLH components of the MBW complex. Indeed, overexpression of SlMYB-ATV in tomato plants abolishes anthocyanin synthesis, and the WT protein can bind to bHLH factors while the mutated form cannot [72]. The expression of *SlMYB-ATV* is directly regulated by the MBW complex, thus establishing a negative feedback loop, as the more the pathway is activated, the more the negative regulator is expressed [72].

Synergistic effects of *atv* and *Aft* alleles allow purple fruit pigmentation in tomato

It became clear that the *atv* and the *Aft* lines could be used to breed commercial purple tomatoes. The loci together demonstrated a strong synergistic effect that elevated anthocyanin content in tomato fruit peel (Figure 3A,B). In fact, the *atv* locus confers an ineffective R3-MYB repressor which, when combined with the active R2R3-MYB activator brought by the *Aft* locus, leads to

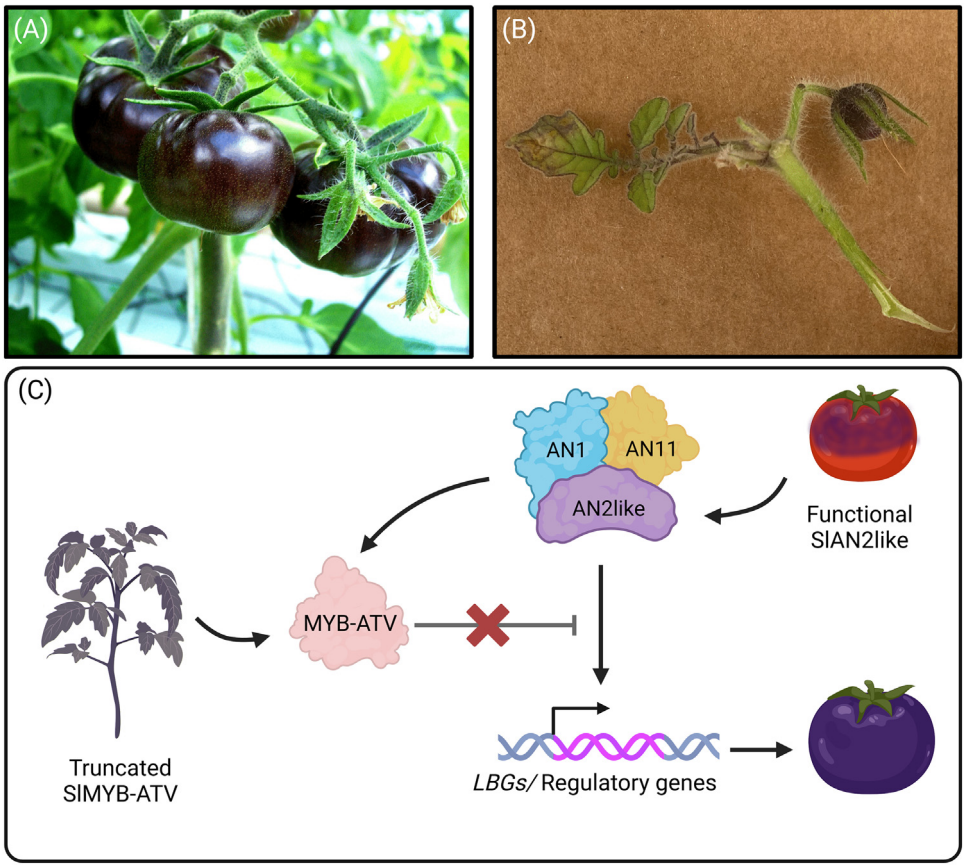

Figure 3. Anthocyanin-enriched tomato fruits are produced by breeding *atroviolacea (atv)* and *Anthocyanin fruit (Aft)* alleles. Anthocyanin-rich fruits from the SunBlack™ variety (A) and a branch of a tomato plant with leaves and a very small purple fruit of *Solanum galapagense* LA 1141 lines (B), both harboring the genetic combinations of functional *SlAN2like* alleles and *atv* mutation. (C) The transcriptional regulation occurring in the fruit peel of the anthocyanin-enriched lines. The *Aft* allele confers the functional R2R3-MYB transcription factor AN2like which takes part, along with the basic helix–loop–helix (bHLH) factor AN1 and the WDR protein AN11, in the MYB–bHLH–WDR (MBW) complex, thereby activating the anthocyanin pathway in the fruit epicarp. The *atv* allele confers a non-working form of the R3-MYB repressor MYB-ATV, whose absence cannot trigger the negative feedback loop usually occurring once anthocyanin synthesis has been activated, thereby allowing a stronger accumulation of the pigments and thus a more uniform purple color on the fruits. The photograph of the *Solanum galapagense* fruit (B) was kindly provided by Dr David Francis [62]. Abbreviation: LBGs, late biosynthetic genes.

a strong and robust anthocyanin synthesis in the fruit epicarp [51,52,77] (Figure 3C). Purple tomatoes originating from this combination need strong light or low temperatures to induce the strongest anthocyanin synthesis and so, to achieve the most intense and homogeneous fruit pigmentations [68]. Interestingly, other metabolites, such as carotenoids, are maintained at the same level as in anthocyanin non-pigmented fruits, meaning that the increased flavonoid synthesis is not metabolically detrimental to carotenoid accumulation [78]. Different commercial lines were thus produced, probably all based on the same mutated alleles of SlMYB-ATV and functional alleles of SlAN2like, such as the Sun Black™ tomato varieties [78,79], Indigo Rose tomatoes[v], V118 [80], Blue Japan Indigo tomatoes [81], and Yoom tomatoes[vi].

Alternative sources of alleles that enhance anthocyanin biosynthesis in fruit have been studied, and *Abg/– atv/atv* plants have been obtained. The combination of *Abg* and *atv* revealed a stronger synergistic effect than the one of *atv* with *Aft,* and fruits increased almost fourfold in the total content of anthocyanins [43]. However, the use of *Abg* allele for the breeding process was not further explored due to issues with the fertility and viability of the homozygous genotypes, likely conferred by the original background of the line or by other characteristics introgressed from *S. lycopersicoides*, which is evolutionarily quite distant from *S. lycopersicum* [43,58].

It is possible that there will be further improvements of these genetic combinations and relative phenotypes, since other alleles of *Aft* and *atv,* as well as of other positive or negative regulators, may exist, such as those recently described in *S. galapagense* [62] (Figure 3B).

Concluding remarks and future perspectives

Enhancing the anthocyanin content in food crops has become a key target in breeding and biotechnology to increase the dietary intake of health-promoting phytochemicals. The tomato lends itself particularly well to such enhancements.

Purple tomatoes have been obtained using transgenic and conventional breeding approaches. The transgenic strategy fully achieved the goal of an anthocyanin-rich tomato by introducing into its genotype strong TF-encoding loci that had already been isolated in other species. Breeders have exploited natural biodiversity and have established the synthesis of anthocyanins in tomato fruit peel through introgression of specific allelic variants from wild relatives.

However, purple tomatoes obtained through conventional breeding have some flaws in their phenotype (see Outstanding questions): they need exposure to strong light to become dark purple, high temperatures repress their anthocyanin content, and often the shaded part of the peel is not uniformly pigmented. To overcome this, it is essential to increase current knowledge on how anthocyanin synthesis is regulated in tomato fruits, with a special focus on photo- and thermomorphogenesis, which are the next two big regulatory mechanisms of pigment accumulation that still need to be fully characterized in this species. Most importantly, the flesh of these tomatoes is not purple and thus is low in anthocyanins. The reasons behind the absence of anthocyanins in the flesh of purple tomatoes need to be fully understood so that they can reach the extremely high anthocyanin contents of transgenic tomatoes and match them in their nutraceutical properties.

Outstanding questions

What is the role of alternative splicing of the factor *SlAN2like* in anthocyanin synthesis, and when did it appear from a phylogenetic point of view? Recent studies showed a novel type of regulation based on AS of this factor in the *Aubergine* line: is this mechanism also conserved in wild relatives of tomatoes?

Synthesis of anthocyanins correlates with the amount of light received by the fruit; shaded parts of the fruits are not able to synthesize such compounds. How does light regulate the synthesis of anthocyanins in tomato?

Exposure to high temperatures dampens the anthocyanin synthesis, while exposure to low temperatures results in a higher anthocyanin content in tomato fruit peel. How does temperature regulate the biosynthetic process in tomato fruits?

How does anthocyanin enrichment of fruit peel prolong the fruit shelf life, and which molecular mechanisms affect sensitivity to unspecialized necrotrophic fungi, such as *Botrytis cinerea*? Are other pathogenic mechanisms also affected by the presence of anthocyanins in the fruit peel?

Why do bred purple tomatoes only accumulate anthocyanins in the epicarp? What is blocking the synthesis in the fruit flesh?

Declaration of interests

No interests are declared.

Resources

[i]www.aphis.usda.gov/aphis/newsroom/stakeholder-info/sa_by_date/sa-2022/purple-tomato)

[ii]www.aphis.usda.gov/brs/pdf/rsr/21-166-01rsr-review-response.pdf

[iii]www.fda.gov/media/170056/download

[iv]https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A52023PC0411

[v]https://today.oregonstate.edu/news/osu-breeding-program-produced-series-purple-tomatoes-healthy-antioxidants

[vi]www.yoomtomato.com/

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