Purple as a tomato: towards high anthocyanin tomatoes

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Anthocyanins are naturally occurring pigments ubiquitously present in plants and, as such, part of the human diet. Owing to their biological activity, anthocyanins have beneficial health effects but, unfortunately, are not present in some important crop plants, such as tomatoes. Recently, a 'purple' tomato, highly enriched with anthocyanins, was produced by the ectopic expression of two selected transcription factors from the ornamental flower snapdragon. In addition to being enriched with anthocyanin, these fruits also prolonged the life of cancer-susceptible mice, suggesting that they have additional health-promoting effects.

Anthocyanins and the nutritional value of food

The colour of flowers and fruits is caused by the presence of different kinds of pigment belonging to the phenylpropanoid and terpenoid classes, whose three major groups are chlorophylls, carotenoids and anthocyanins. In some crops, domestication has possibly resulted in selection for agronomic traits that prevail over colour characteristics, whereas in others, an increase or refinement in the pigments naturally present in the edible tissues has occurred. This is likely to apply to tomato (*Solanum lycopersicum* L.), the fruit of which is rich in several carotenoid pigments, such as lycopene and phytoene [1], but contains only small quantities of some flavonoids; in addition, and somewhat surprisingly, the fruits do not usually produce anthocyanins [2], unlike the fruit of other Solanaceae, such as eggplant (*Solanum melongena* L.) or pepper (*Capsicum* spp).

Vegetables, fruits and plant-derived food, such as bread, are fundamental components of the human diet, and the relative content of compounds that are beneficial to human health is an important subject. Among these, considerable importance is given to the presence of some secondary plant metabolites, such as anthocyanins and other flavonoids, which, owing to their health-promoting effects [3–5], add nutritional value to the plant-derived foods that contain them. As a result, in recent years, an increasing number of different approaches have been adopted to stimulate the synthesis of flavonoids in plants [6,7]. Although most of these studies were carried out in a few model species [6], some crops, such as potato and rice, containing suboptimal amounts of these compounds, have also been genetically modified to increase their flavonoid content [8,9]. Some such studies focused also on tomatoes,

as these are among the most important vegetables worldwide. Recently, the expression in tomato of two selected transcription factors (TFs) involved in anthocyanin production in snapdragon (*Antirrhinum majus* L.) led to high levels of these flavonoids throughout the fruit tissues, which, as a consequence, were purple coloured [10]. This result demonstrates that the anthocyanin biosynthetic pathway can be fully switched on in fruit of this species if activated appropriately.

Why do tomatoes not synthesize anthocyanins naturally?

Anthocyanins are an important group of naturally occurring polyphenolic compounds derived from the phenylpropanoid biochemical pathway (Box 1) [11,12]. They are members of the flavonoid class of plant secondary metabolites, whose common basal structure consists of two aromatic benzene rings separated by an oxygenated heterocycle, including three carbon atoms [12]. In the glycosylated forms, anthocyanins are soluble pigments that are stored in the acidic vacuole of cells in flowers and fruits (particularly berries), where they are synthesized to attract pollinators and animals for seed dispersal [11,12]. They can also be produced in vegetative tissues in response to stressful events, such as high irradiance, against which they can give protection, acting as both a light screen and scavengers for radical species [13]. The presence of anthocyanins in plant-derived food is also important because their intake in the human diet is associated with protection against coronary heart disease and an improvement in sight [4]. They might also prevent cholesterol-induced atherosclerosis, could have anti-inflammatory and anticarcinogenic activities and could aid in the prevention of obesity and diabetes [4]. The biological effects of anthocyanins and flavonoids have been classically ascribed to their antioxidant activity [3]; however, a more recent emerging view is that they might exert modulatory effects, affecting signalling pathways in animal cells [5].

Tomato plants contain a variety of flavonoids in their vegetative tissues, including anthocyanins [14]. In the fruit, they accumulate only small amounts of naringenin chalcone (a flavonoid biosynthetic pathway intermediate) and some flavonols, such as quercetin and kaempferol glycosides, which are concentrated in the peel but do not synthesize anthocyanins [2,14]. 'Black' or 'purple' fruits often described in some heirloom varieties result from mutations affecting chlorophyll breakdown and carotenoid content but are not related to anthocyanin production [14]. By contrast, the production of anthocyanins in the fruit is

retained by some taxonomically close wild *Solanum* species, such as *Solanum chilense* Dunal (Figure 1a) [15]. Interspecific crosses with wild species transferred the ability to produce small quantities of anthocyanins into the peel of cultivated tomatoes. For example, the dominant gene *Anthocyanin fruit* (*Aft*), which induces limited pigmentation upon stimulation by high light intensity, was introgressed into domesticated tomato plants by an interspecific cross with *S. chilense* (Figure 1b) [14,15]. Similarly, the gene *Aubergine* (*Abg*), which was introgressed from *Solanum lycopersicoides* Dunal, can induce a strong and variegated pigmentation in the peel of tomatoes [14,15]. Furthermore, the recessive gene *atroviolacea* (*atv*), derived from the interspecific cross with *Solanum cheesmaniae* (L. Riley) Fosberg, has been shown to stimulate strong anthocyanin pigmentation in the entire plant, particularly in vegetative tissues (Figure 1c) [14]. Fruits with either *Aft* and *atv* alleles (Figure 1d,e) or *Abg* and *atv* alleles have been obtained and have generally shown a higher production of anthocyanins in the peel, ranging in total amount from 1 to 4 mg g^{-1} fresh weight of peel [14].

The flavonoid biosynthetic pathway has been analysed in different *S. lycopersicum* lines [16], including *S. lycopersicum* v. *cerasiforme*, which is the subspecies thought to be the most probable wild ancestor of domesticated tomatoes [17]. A major constraint found in the flavonoid biosynthetic pathway is the lack of expression of the *chalcone isomerase* (*CHI*) gene (Box 1, Figure Ia) in the peel of the fruit, which is probably caused by a mutation in a fruit-specific element of the promoter [16]. This could explain the high level of naringenin chalcone in this tissue of tomatoes, because this is the main substrate of the CHI enzyme (Box 1, Figure Ia). The biosynthetic pathway, by contrast, seems to be constitutively switched off in the flesh of the fruit, where none of the structural genes analysed are expressed [16]. Reintroducing the expression of *CHI* in cultivated tomato fruit, by interspecific crosses with wild tomato species [16] or heterologous expression of *CHI*

Box 1. The anthocyanin biosynthetic pathway

Anthocyanins are synthesized through a branch of the flavonoid biosynthetic pathway (Figure Ia) (reviewed in Refs [11,12]). Genes involved in the pathway can be grouped into two different classes: those encoding the enzymes that catalyse the different reaction steps (structural genes) and those regulating the expression of the structural genes (regulatory genes). The enzymatic reactions of flavonoid biosynthesis and most of the corresponding structural genes have been identified and cloned in several species, including cultivated tomato plants, where many of them have been mapped [32]; most of the relative EST (expressed sequence tag) sequences are deposited in GenBank or in the Sol Genomic Network database (http://www.sgn.cornell.edu).

Regulatory genes encode TFs and other regulatory proteins, which interact with the promoter regions of the structural genes, enabling precise temporal and spatial coordination of their transcription and of the resulting production of anthocyanins. Several studies indicate that two main classes of TFs are involved in anthocyanin production: R2R3 MYB-type and bHLH-type TFs [31]. Recent models indicate that these physically interact and, with the participation of another protein with a 'WD40' repeat (WDR), form a MYB–bHLH–WDR (MBW) transcription complex that is directly responsible for the activation of the structural gene transcription (Figure Ib) [31].
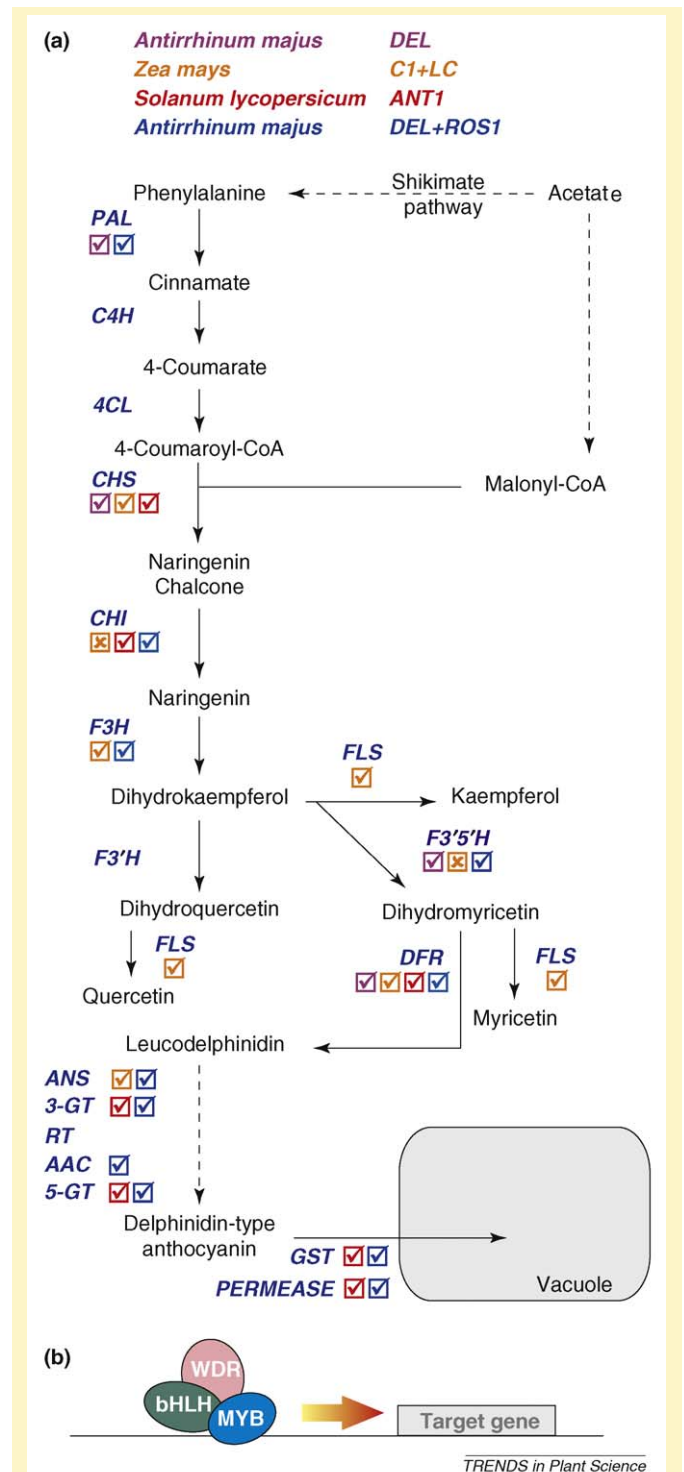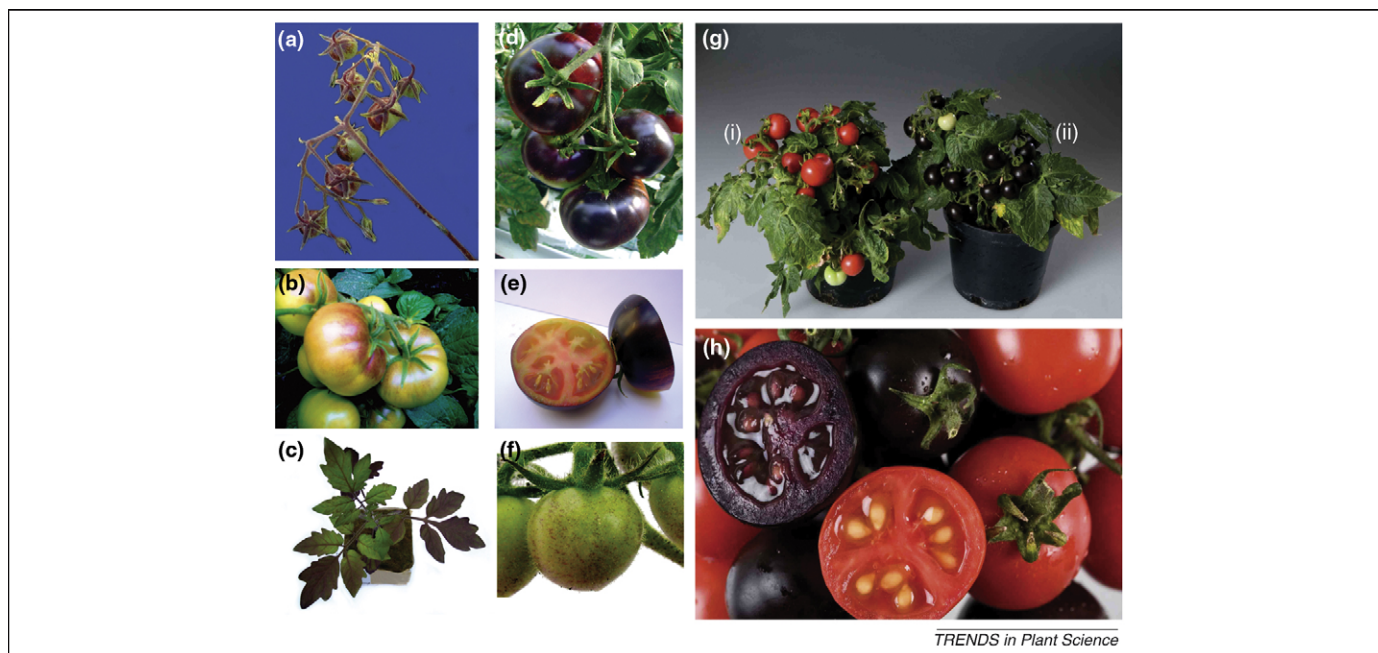

Figure I. Anthocyanin biosynthetic pathway and its regulation. **(a)** Anthocyanin biosynthesis in tomato plants. Only the production of delphinidin-type anthocyanins, representing the major class observed in tomato plants [23], is shown. Enzyme abbreviations: PAL, phenylalanine ammonia-lyase; C4H, cinnamate-4-hydroxylase; 4CL, 4-coumaroyl:CoA-ligase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3'5'H, flavonoid 3'5'-hydroxylase; FLS, flavonols synthase; DFR, dihydroflavonol 4-reductase; ANS, leucoanthocyanidin dioxygenase; 3-GT, 3-*O*-glucosyltransferase; RT, rhamnosyl transferase; AAC, anthocyanin acyltransferase; 5-GT, 5-*O*-glucosyltransferase; GST, glutathione S-transferase; PERMEASE, putative anthocyanin transporter. The TFs that have been expressed in tomato plants are listed at the top of the figure and the corresponding effects on the activation (✔) or lack of effect (✘) of the main target genes of the pathway (when analysed) are reported, step by step, using coloured symbols. **(b)** The MBW (MYB–bHLH–WDR) transcription complex responsible for the activation of the structural flavonoid genes [31].

Figure 1. Overview of anthocyanin-enriched tomatoes. **(a)** *Solanum chilense* Dunal fruit [15]. **(b)** *Solanum lycopersicum* fruit with the *Aft* gene [15]. **(c)** *S. lycopersicum* plant with the *atv* gene [14]. **(d,e)** *S. lycopersicum* fruit with both the *Aft* and *atv* genes [14] (*Aft* × *atv* plants obtained by Gian Piero Soressi). **(f)** Fruit of *S. lycopersicum* cv. MicroTom overexpressing *AtMYB75* [25]. **(g)** Comparison of an *S. lycopersicum* cv. MicroTom control plant (i) with an *AmDel/AmRos1*-overexpressing line (ii) [10] and **(h)** sections of the fruit. Reproduced with permission from Roger Chetelat (a); and Andrew Davis and Sue Bunnewell (g,h).

isolated from other species, for example from *Petunia* [18], results in tomato plants with higher levels of quercetin glycosides in their fruit peel. This shows that, when removing the bottleneck of CHI in the biosynthetic pathway, flavonoids can be synthesized. It is therefore likely that the default state for the peel of tomatoes is to produce these phenolic compounds, as hypothesized [16].

It was also suggested that cultivated tomato might have lost *CHI* expression in the fruit peel relatively early during domestication or even before, given that this trait was observed in all the cultivars of domesticated tomato analysed, including *S. lycopersicum* v. *cerasiforme* [16]. It is tempting to speculate that, because the fruit peel of some wild tomato species and of other Solanaceae (e.g. eggplant [19] and pepper [20]) can accumulate anthocyanins, other natural mutations occurred in regulatory and/or structural genes of the flavonoid biosynthetic pathway, affecting not only flavonol production but also anthocyanin synthesis in tomatoes [21]. Certainly, further studies are necessary to clarify this point.

Engineering anthocyanin accumulation in tomato fruit: towards a 'purple' tomato

Recently, various transgenic approaches have been taken to increase flavonoid levels in tomato fruit by overexpressing either the structural or regulatory genes involved in the biosynthetic pathway (Box 1). Most of these attempts have exploited heterologous genes [18,22–26], and in only one case was an endogenous tomato regulatory gene (*ANT1*, encoding a MYB-type TF [Box 1]) overexpressed in tomato plants [27]. Although a significant increase in the final content of some flavonoids (flavonols in particular) was obtained in several cases [18,23,24,26], only partial success was achieved in relation to the production of anthocyanins. For example, the overexpression of the basic

helix–loop–helix (bHLH) TF (Box 1) *Delila* (*Del*) of snapdragon induced strong pigmentation only in the vegetative tissues of the plant [22], whereas the overexpression of both tomato *ANT1* [27] and *Arabidopsis PRODUCTION OF ANTHOCYANIN PIGMENT1* (*PAP1*, also called *MYB75*) (Figure 1f) [25] MYB-type TFs induced only a partial, spotted pigmentation, mostly limited to the peel of the fruit. Whereas the lack of activation of some structural genes was the major cause when no anthocyanins were produced (Box 1, Figure Ia), in the case of partial pigmentation, the low availability of flavonoid biosynthetic precursors or the absence of other limiting regulatory factors (e.g. bHLH TFs) could explain the failure to activate full pigmentation.

Alternative approaches to improving the nutritional value of tomato fruit have also been taken. In particular, manipulation of genes encoding components of the light-signalling transduction machinery can affect the final content of secondary metabolites, as shown by the fruit-specific RNA interference (RNAi)-mediated suppression of *DE-ETIOLATED1* (*DET1*), a photomorphogenesis regulating gene. Inactivation of *DET1* led to a significant increase in both flavonoids and carotenoids in tomato fruit [28].

These constraints encountered when trying to obtain high-anthocyanin tomato fruits were overcome in the most recent attempt to enrich tomato fruit with anthocyanins, by Eugenio Butelli *et al.* [10]. These authors selected two different snapdragon TFs – *Del*, which is a bHLH-type TF, and *Rosea1* (*Ros1*), an R2R3 MYB-type TF – and overexpressed them in tomato (cv. MicroTom) under the control of a fruit-specific E8 promoter. Whereas transgenic plants showed normal vegetative growth, transgenic fruit started to synthesize anthocyanins at the end of the green stage and continued to accumulate these pigments during subsequent ripening, ultimately reaching an intense, uniform

purple colouration both in the peel and in the flesh (Figure 1 g,h). In the fruit of the line with the strongest visible pigmentation, the final concentration of anthocyanins averaged ~3 mg g^{-1} fresh weight [10], which is the highest value obtained so far in this species.

This striking phenotype was due to a combination of factors. First, *Del* and *Ros1* stimulated the transcription of most of the structural genes involved in the biosynthetic pathway, including *phenylalanine ammonia-lyase* (*PAL*), *CHI* and *flavonoid 3'5'-hydroxylase* (*F3'5'H*) [10]. *PAL* induction is necessary to ensure high levels of flux through the general phenylpropanoid metabolism to feed flavonoid biosynthesis [29], whereas *CHI* and *F3'5'H* are both essential to address the flux of flavonoid intermediates towards the anthocyanin products (Box 1, Figure Ia). *PAL*, *CHI* and *F3'5'H* were not induced when other heterologous anthocyanin-related TF genes, such as maize (*Zea mays* L.) *Leaf colour* (*LC*) and *Colourless1* (*C1*), were expressed in tomato plants, resulting in a higher levels of flavonols, but no anthocyanins [23]. Such a difference confirms that *F3'5'H* expression is necessary to activate anthocyanin synthesis in tomatoes [23] because only delphinidin-type anthocyanins, which derive from dihydromyricetin and therefore require F3'5'H enzymatic activity (Box 1, Figure Ia), are produced in these plants. At the same time, stronger activation of the entire phenylpropanoid pathway, caused by the significant increase in both *PAL* transcription and PAL enzymatic activity and in the expression of *CHI*, seems to be important to obtain very high levels of anthocyanins, such as those observed in *Del–Ros1* transgenic plants [10].

Second, *Del* and *Ros1* also stimulated genes involved in the side-chain modification of the anthocyanin pigments and genes possibly related to the final transport of these molecules into the vacuole [10], processes that are both necessary for the accumulation of anthocyanin (Box 1, Figure Ia). Therefore, although TFs from different plant species are involved in the same biosynthetic process, they are characterized by a different specificity in their target genes, which might be particularly marked in heterologous host species [30]. A sustained expression of both bHLH and MYB TFs (Box 1, Figure Ib) is also necessary to obtain a uniform and widespread synthesis of pigments within the fruit.

The high production of anthocyanins in these 'purple' tomatoes was not obtained at the expense of other major classes of tomato fruit pigments, and the antioxidant activity attributable to the carotenoid fraction remained unchanged [10]. This could be due to the careful selection of the promoter (E8) for expression of regulatory genes. Expression late in fruit development meant that anthocyanin biosynthesis did not interfere with other metabolic processes occurring earlier in fruit ripening. These tomatoes therefore fulfil the requirements in terms of total (lipophilic [from carotenoids] + hydrophilic [from flavonoids]) antioxidant activity.

Importantly, in a pilot experiment, a group of cancer-susceptible mice that were fed a diet supplemented with these high-anthocyanin tomatoes showed a significant extension of their average life span compared with cancer-susceptible mice that were fed a diet supplemented with regular control tomatoes [10]. This demonstrates how an anthocyanin-enriched tomato-derived food can be beneficial for animal health. Further studies are clearly required to confirm the potential beneficial role of these anthocyanin-rich tomatoes in human health.

Perspectives

The work of Butelli *et al.* [10] and previous related papers highlight the interest in the production of crop plants enriched with health-promoting compounds. Among these, anthocyanins and flavonoids are some of the most important targets for improving the nutritional value of foodstuffs. Extensive knowledge of the molecular mechanisms underlying the elaborate and sophisticated biosynthetic pathways of secondary metabolites in plants is essential to drive metabolic engineering strategies in the most rewarding direction, as Butelli *et al.* [10] have shown.

Furthermore, given that the biological properties of different anthocyanin compounds and of the other classes of flavonoids are often molecule-specific, a major objective of metabolic engineering approaches might be not only increasing but also optimizing their levels and composition in crops. This could be done, for example, by combining the use of regulatory and structural genes. Additionally, researchers could exploit natural genetic resources, either as an alternative to genetic engineering approaches or to strengthen them. The identification of the complete set of endogenous regulatory proteins involved in flavonoid metabolism in tomato and its close wild species, in particular the characterization of the mutations *Aft*, *Abg* and *atv*, will thus contribute in this sense to the production of high nutritional value tomatoes. Further work is also needed to investigate the potential health benefits of these unusual, but colourful, tomatoes.

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