# Engineering anthocyanin biosynthesis in plants

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Anthocyanins are water-soluble pigments responsible for the red, purple and blue colours of many flowers and fruit. In addition to their physiological roles in plants, to attract pollinators and seed dispersers, dietary anthocyanins are associated with protection against certain cancers, cardiovascular diseases and other chronic human disorders. Enhanced supplies of pure anthocyanins would service the demands of research to investigate these health-promoting effects and would also prove a valuable resource for the colourants and cosmetic industries to investigate the effects of chemical modifications, co-pigments, and pH on colour and stability for developing new plant sources of natural colourants, and new natural colours.

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## Introduction

As water-soluble, natural pigments, anthocyanins are responsible for the red, purple and blue colours of many flowers and fruit, that attract pollinators and seed dispersers [1]. Anthocyanin production is also induced under stress conditions [2-6] or infection by pathogens [7] and may serve to protect plants against oxidative damage such as that caused by high irradiation when the capacity for carbon fixation is low. Anthocyanins protect photosynthetic tissues by absorbing light in the visible range, so reducing light stress, and also by serving as antioxidants.

In addition to their physiological roles in plants, dietary anthocyanins are associated

with protection against certain cancers [8], cardiovascular diseases [9], as well as other chronic human disorders [9]. Although many studies attribute the protective effects of dietary anthocyanins to their antioxidant capacity, their bioavailability is low and they likely promote health by suppressing specialised signalling pathways involved in inflammation and disease development [10]. Enhanced supplies of pure anthocyanins would service the demands of research to investigate these effects and would also prove a valuable resource for the colourants and cosmetic industries to investigate the effects of chemical modifications, co-pigments, and pH on colour and stability for developing new plant sources of natural colourants, and new natural colours.

There are therefore excellent reasons for engineering anthocyanin biosynthesis in plants for a diversity of applications. Understanding the enzymes that determine their chemical features and the factors that influence their functional properties, should lead to more efficient breeding efforts to generate novelty in colours and patterns as well as to the engineering of colour changes beyond the limits of natural variation.

**Synthesis of anthocyanins**

Anthocyanins are synthesized from three molecules of malonyl CoA derived from fatty acid metabolism and one of p-coumaroyl CoA synthesized from phenylalanine via the general phenylpropanoid pathway (Figure 1). Malonyl CoA and p-coumaroyl CoA are condensed by chalcone synthase, the first committed enzyme of flavonoid biosynthesis. The activity of four further enzymes in flavonoid biosynthesis (Chalcone Isomerase [CHI], Flavanone 3-Hydroxylase [F3H], Dihydroflavonol 4-Reductase [DFR] and Anthocyanidin Synthase [ANS, also known as LDOX]) are required to synthesise a coloured anthocyanidin (pelargonidin) (Figure 1). However most plants further hydroxylate the B-ring using either the enzyme Flavonoid 3'Hydroxylase [F3'H] which forms cyanidin, or the enzyme Flavonoid 3'5' Hydroxylase [F3'5'H] which forms delphinidin. Differences in the hydroxylation of the B-ring confer differences in the colour range of anthocyanidins, pelargonidin being orange to red, cyanidin red to red-purple and delphinidin being red-purple to

blue depending on many other factors. Anthocyanidin structure is inherently unstable and in nature these pigments accumulate exclusively as glycosylated forms (anthocyanins) where carbon 3 is linked through oxygen to a sugar residue, which is most frequently, but not exclusively, glucose. Post synthesis, cyanidin may be methylated on its 3' hydroxyl group to form peonidin, and delphinidin may be methylated on its 3' hydroxyl group to form petunidin or on both its 3' and 5' hydroxyl groups to form malvidin. Pelargonidin, cyanidin, peonidin, delphinidin, petunidin and malvidin constitute the core anthocyanidins predominant in nature. The 600+ natural anthocyanins identified to date differ in their side chain decorations of these core anthocyanidins. Side chain decorations affect anthocyanin functionalities including: colour, stability, interactions with other compounds, bioavailability and possibly also their health promoting effects. Engineering the types of anthocyanins that accumulate in plants involves modification of these decorations.

Anthocyanins change colour depending on the prevailing pH, because they exist in four pH-dependent forms shown in Figure 2. It is the lack of stability in the colour of anthocyanins, especially at neutral and high pH that causes the main problem in the uses of anthocyanins, particularly as natural food colourants. However, plants have solved many of these stability problems by elaborating the decoration of their anthocyanins, adjusting the vacuolar pH and adopting co-pigmentation strategies to generate an enormous range of orange through blue anthocyanin-based colours, particularly familiar in flowers.

**Decoration of anthocyanins: glycosylation**

Glycosylations are usually catalyzed by cytoplasmically-localized glycosyl transferases, and the most common sugars in anthocyanidin 3-monosaccharides are glucose, rhamnose, galactose, arabinose and xylose, in descending order of occurrence. Glycosylation of C3 occurs on all naturally-accumulating anthocyanins and increases the stability of the pigment [11]. Further glycosylation shifts colour slightly towards red. Common sugars in anthocyanin 3-biosides are rutinose (glucose

plus rhamnose), sophorose (glucose plus glucose) and sambubiose (glucose plus xylose) [12,13] and 3-biosides are generally more stable than anthocyanidin monosides [14]. The enzymes adding the sugar residues to the different positions of the anthocyanidins belonging to distinct clusters of family 1 glycosyl transferases. 3,5 diglycosides are also common and while glycosylation of the 3 position significantly improves the stability of anthocyanins compared to anthocyanidin aglycones, glycosylation of the 5 position may reduce stability. At any given pH, anthocyanin 3-glucosides are more coloured than the equivalent anthocyanin 3,5 diglucosides because the loss of an OH group at the C5 position accellerates the hydration reactions which lead to the formation of colourless pseudobases (Figure 2) [11,14]. Other residues of the anthocyanidins that can be glycosylated are the 7 position and the 3' and 5' positions of the B-ring. Additional glycosylations provide more sugar residues for decoration with acyl groups, both aromatic and aliphatic, which affect colour and increase the stability of the pigments.

Some glycosylation reactions do not use sugar nucleotides but instead use acyl sugars as donors. These reactions are catalyzed by enzymes belonging to the family 1 glucose hydrolase proteins [15] and have been characterized in delphinium and carnation. They likely act late in biosynthesis following transport of anthocyanins already decorated, into the vacuole. The activity of these types of glycosyl transferases may be restricted to specific genera or individual species with specialized decoration of their anthocyanins.

**Decoration of anthocyanins: acylation**

The sugar residues of anthocyanins are often acylated with aromatic acids (*p*-coumaric, caffeic, ferulic, sinapic, gallic or *p*-hydroxybenzoic acids) or aliphatic acids (malonic, acetic, malic, succinic, tartaric and oxalic acids) (Figure 3). Acylation of anthocyanins increases their stability, through the processes of intra- and intermolecular co-pigmentation. In some plants the acyl groups are, themselves, glycosylated. Some of the most complex anthocyanins have alternating glycosyl and

acyl groups, which generally leads to increased stability of these anthocyanins in solution (Figure 3). Aromatic acylation additionally shifts the colour of the pigments towards the blue, and some of the most intense blue colours of flowers such as those found in morning glory and lobelia are conferred by poly-acylated (aromatic) anthocyanins [16,17].

Acylation is most often catalysed by BAHD acyltransferases using Acyl CoA donors [18]. These enzymes are active in the cytoplasm. Acyl groups added to anthocyanins late in decoration, may be added in the vacuole in reactions catalysed by serine carboxypeptidase-like proteins [19].

At higher pH values (6-6.5), the anthocyanin quinoidal bases (which are blue) form. These can be stabilised through processes termed 'co-pigmentation', either intramolecular or intermolecular. In intramolecular co-pigmentation, the conversion to the carbinol pseudo-base/chalcone, which involves hydration, is inhibited such that the formation of quinoidal bases from the flavylium ions is favoured, and colour is stabilised and shifted towards the blue (Figure 2). Intramolecular co-pigmentation involves side chain decorations of anthocyanins, particularly aromatic acyl groups which stack, sandwich-like, with the ring structures of the anthocyanins (known as π stacking). These molecular stacks are promoted by intramolecular bonding. Consequently, glycosylation is required for stability both in its own right and because acylation occurs on the glycosyl groups. Aliphatic acylation promotes stability of anthocyanins but does not cause a bathochromic shift and may not involve π-stacking [14].

**Intermolecular co-pigmentation.**

In intermolecular co-pigmentation, quinoidal bases are stabilised by stacking, but this time with other molecules, often other flavonoids such as flavones, flavonols, other compounds with aromatic rings (hydroxycinnamates, alkaloids, amino acids, organic acids, and other anthocyanins), nucleosides and polysaccharides [20]. Intermolecular co-pigmentation results in a hyperchronic shift in absorption by the anthocyanin, so

that the pigment appears more intense, and a bathochromic shift such that the absorption maximum is increased and the pigment appears bluer, even though the co-pigment itself is uncoloured. The maximal effects of co-pigmentation are observed at pH 3.6 although the prevailing pH of the vacuole is more usually between 5.2 and 5.5 in most plant cells.

**Regulators of anthocyanin biosynthesis.**

The control of anthocyanin biosynthesis is highly conserved in angiosperms and likely also in gymnosperms. Members of three protein families, R2R3MYB transcription factors, bHLH transcription factors and WD Repeat Proteins (WDR) interact to form a complex (MBW) that activates anthocyanin biosynthesis. The WDR proteins of the MBW complex are thought to serve a stabilising function and probably interact directly with the bHLH proteins. They are not thought to bind DNA. The bHLH transcription factors (TFs) involved in the MBW complex belong to conserved sub-families [21] characterised by a conserved N-terminal domain that interacts with the MYB partners in the MBW complex [22]. The bHLH partners in the MBW complex may have independent functions additional to their regulation of anthocyanin biosynthesis, for example in the regulation of proanthocyanidin biosynthesis, in the control of trichome and root hair formation in Arabidopsis, and in the control of vacuolar pH and seed coat morphology in Petunia [22-26]. The broad range of biological functions of the bHLH partners means that their activity usually does not limit anthocyanin accumulation in different cell types, and they may be expressed in epidermal cells in which anthocyanin is not being produced. The activity of the R2R3MYB regulators in controlling anthocyanin biosynthesis is much more specific. Usually, species have multiple copies (paralogues) of these genes, which confer anthocyanin production in different patterns or cell types. It is the activity of the MYB TFs that normally determines the amount of anthocyanin produced by specific cells, and so differences in flower colour intensity and pattern are usually attributable to differences in the expression of the MYB TFs in the MBW complex. These MYB TFs belong to subgroup 6 of the R2R3MYB family and carry a highly conserved

signature motif within their DNA binding domain [22] which is predictive of and functions in the interaction with subgroup IIIf bHLH proteins. They also carry a conserved motif in their C-terminal domains, (termed the anthocyanin box) which is characteristic of most R2R3MYB proteins regulating anthocyanin biosynthesis through the MBW complex [27]. The R2R3MYB proteins of the MBW complex bind to DNA and are thought to be the principle determinants of the target genes that the MBW complex activates. The R2R3MYB proteins regulating anthocyanin biosynthesis in monocotyledonous plants such as maize are more closely related structurally to the R2R3MYB regulators of proanthocyanidin biosynthesis than to the dicot R2R3MYB anthocyanin regulators. Consequently, monocot MYBs do not work well in dicots [28] and similarly dicot MYBs do not work well in monocots, in inducing anthocyanin biosynthesis [29].

Other small proteins with single copies of the MYB repeat, can not bind DNA selectively but act as negative regulators of anthocyanin biosynthesis by interaction with the bHLH proteins, competitive with the R2R3MYB activators. The small size of these competitive repressors means that they can move from cell to cell and can repress anthocyanin biosynthesis non-cell autonomously [30].

Two points are of importance to the metabolic engineering of anthocyanin biosynthesis using TFs. The first is that activation of the pathway is obligately dependent on the activity of all three classes of protein within the MBW complex. Despite this, many groups have been able to induce anthocyanin biosynthesis ectopically by expression of the MYB protein [31,32] and this has led to the impression that activation of the MYB protein alone is necessary to increase anthocyanin production [33]. The WDR protein is likely expressed constitutively [34] and the bHLH proteins may be expressed in tissues where anthocyanins are not produced, possibly due to their diverse regulatory roles [23]. Consequently the activity limiting anthocyanin accumulation is, most often, the specialised MYB TF. The contribution of bHLH proteins to induction of anthocyanin biosynthesis can be

judged by comparing pigmentation induced by high levels of both the MYB and the bHLH proteins [35,36] to that induced by high levels of the MYB protein, alone [36-39]. Combined induction of both MYB and bHLH proteins leads to levels of anthocyanins equivalent to those in the darkest berries (3-6 mg per gram FW). This is equivalent to 3-5% dry weight accumulating as anthocyanins and may represent a maximum level compatible with sustained plant cell viability.

The second consideration of importance to metabolic engineering involves the targets of the MBW regulatory complex. Analysis of mutants of anthocyanin MBW TFs has suggested that their targets may be different in different plant species; for example the MBW complex has been reported to regulate expression of the genes encoding the enzymes of the entire pathway committed to flavonoid biosynthesis in kernals of maize [40], but only the 'late biosynthetic genes' (LBGs) in dicot flowers. What is classified as an LBG may vary from species to species [41-45]. In contrast, over expression of the proteins of the MBW complex usually results in elevated expression of all the genes encoding enzymes committed to anthocyanin biosynthesis as well as transporters and transport-associated proteins [32,35,36]. The resolution of this paradox probably lies in the way that target genes are identified: target genes show significant reduction in expression in mutants of the genes encoding TFs of the MBW complex. In flowers, alongside the proteins regulating anthocyanin biosynthesis, other TFs controlling flavone and flavonol biosynthesis may also be active [46,47]. These proteins activate the early steps in flavonoid biosynthesis, shared with anthocyanin biosynthesis. Consequently the loss of activity of the MBW complex does not result in down-regulation of these early biosynthetic genes (EBGs), because their activation is complemented by the independent regulators of flavone and flavonol biosynthesis. Thus, the MBW complex can likely induce the entire pathway for anthocyanin accumulation.

Because expression of both MYB and bHLH partners of the MBW complex can induce all steps in the biosynthetic pathway, these proteins are powerful tools for metabolic engineering of a pathway encoded by most higher plants.

**Metabolic engineering of anthocyanins by genetic modification**

Engineering paler colours has been achieved relatively easily by silencing structural genes in the anthocyanin biosynthetic pathway (reviewed in [48]). Shifts in colour from blue to red have been achieved by silencing F3’5’H (Table 1 in [48]).

Some species lack particular anthocyanins because they lack the genes encoding specific decorating enzymes. This deficiency can be overcome by introducing the genes from species that do have them. The best known examples have involved introduction of the F3’5’H gene from petunia and viola into species such as carnation and rose to give flowers of blue-purple colour due to the production of delphinidins [49,50]. While the new F3’5’H gene is usually functional, the DFR enzyme of the host species may be specialized to accept dihydroflavonols with just one or two hydroxyl groups on the B-ring, so flowers containing higher levels of blue-ish delphinidin-based pigments have been produced by replacing the endogenous DFR with DFR from Petunia. DFRs from Petunia or iris have a preference for tri-hydroxylated dihydromyrecetin as their substrate [51]. Two recent papers describe very similar strategies to produce blue/blue-violet chrysanthemums (Figure 4A-D) [52,53]. In chrysanthemum, differences in the ability of F3’5’H enzymes from different plant sources to compete with endogenous F3’H have been observed, meaning that transformations have been undertaken in lines with low F3’H activity (either natural or engineered) to enhance delphinidin production, further.

In gentian, new red-purple flowers have been engineered by inhibiting the acylating activity of an 5,3’ acyl transferase [54]. Reducing the amount of diacylated delphinidin-based anthocyanins reduces intramolecular co-pigmentation so giving a redder colour. Inhibition of flavonol synthase in lisianthus (*Eustoma grandiflorum*) resulted in redder flowers [55] by inhibiting intermolecular co-pigmentation of

anthocyanins by flavonols.

Novel, red coloured seeds of soybean have been produced by inhibiting the activity of anthocyanidin reductase (ANR) in the seed coat [56]. ANR removes anthocyanidins to supply epicatechin for proanthocyanidin synthesis. In the absence of ANR activity, red cyanidin-based anthocyanins accumulate in the seed coat. This new colour has been proposed as a marker for seed from transgenic soybean lines.

**Obtaining higher levels of anthocyanins via metabolic engineering**

The greatest enrichment in anthocyanins has been engineered in tomato by combining expression of an R2R3MYB protein (Rosea 1) and a bHLH protein (Delila) from snap dragon [35]. Key to the success of engineering these tomatoes was expression of the regulatory genes under the control of a fruit specific promoter (from the E8 gene of tomato) that is switched on at breaker once fruit have reached full size and most of the metabolism underpinning fruit development and flavor has been completed. The switching on of anthocyanin biosynthesis late in fruit development has meant that cultivation of purple tomatoes incurs no yield penalty and high levels of anthocyanin accumulate in fruit (about 3% of dry weight as anthocyanins). Levels are high enough for the fruit to be used in nutritional interventions with mouse models of disease. Consumption of rodent pellets supplemented at 10% (dry weight) with purple tomato powder, resulted in cancer-prone animals living 30% longer than those on a standard diet or a diet supplemented at 10% with regular tomato powder [35]. Similar metabolic engineering strategies could be used to make ‘model foods’ with enhanced levels of different phytonutrients, in a common food matrix, such as tomato. Such materials could be used to assess the relative benefits of individual phytonutrients in a common food matrix (comparative nutrition studies).

Purple tomatoes can provide a source of anthocyanins for applications other than consumption. Scale-up is relatively easy in commercially cultivated crops (due to the availability of existing infrastructure for cultivation) and relatively cheap. Industry is also looking for systems for batch production of anthocyanins. A lot of research has

been devoted to establishing cell cultures that stably produce anthocyanins. Despite some success, most such attempts have been confounded because anthocyanins are the products of differentiated cells, and yet suspension cultures are essentially dedifferentiated cells. Consequently, cultures developed for anthocyanin production in the past have proved unstable, long term, and these production systems remain largely uncommercialised. Expression of regulatory genes controlling anthocyanin biosynthesis in suspension cultures results in cells that produce exceptionally high levels of anthocyanins, which can be maintained stably by selecting with antibiotic to prevent silencing of the transgenes (Figure 4E). The cultures yield substantially more (~20mg/g dry weight) anthocyanins than conventional cultures (1-2mg/g dry weight) and are stable over long periods of culture. These methods work well for tobacco and tomato cell cultures, but are extendable to other species which carry the genetic information to produce very exotic anthocyanins, such as grape, purple sweet potato or the multiply glycosylated and acylated anthocyanins of the 'Heavenly Blue' morning glory. High level production of such specialized anthocyanins is of considerable interest for providing material for assays of stability and colour determination, for new natural colour formulations, and ultimately for the production of new colours as well as providing anthocyanins for bioavailability and bioefficacy studies.

**New applications resulting from engineering anthocyanins in plants**

In addition to their classic roles in modifying flower colours and health benefits in food, recent research has shown that enrichment with anthocyanins can extend the shelf life of tomato fruit by delaying over-ripening and reducing the susceptibility to of fruit to the postharvest, necrotrophic pathogen, *Botrytis cinerea* (Figure 4F) [57,58]. As antioxidants, anthocyanins increase the anti-oxidant capacity of crops, and thus can reduce the oxidative damage that occurs during over-ripening and pathogen infection. Due to the high antioxidant capacity of anthocyanins, enriched fruit can also serve as an excellent production system for recombinant proteins [59].

The distinct appearance of anthocyanin pigmentation also makes it a very good visual

marker for biological research: anthocyanin can be used as a visible marker for plant transformation [60] and viral infection [61]. Alternatively, co-suppression of candidate genes and anthocyanin biosynthetic/regulatory genes in anthocyanin-enriched plant tissue provides an excellent marker for viral-induced gene silencing [58,62].

**Is using plants to produce anthocyanins better than using bacteria or yeast?**

Although progress has been made in engineering anthocyanin production in microbes, recently [63], using plants or plant cell cultures to produce anthocyanins remains the most efficient strategy. The anthocyanin biosynthetic pathway is present in most plant species, which, consequently, are capable of making anthocyanins in certain tissues or under certain conditions. Unlike microbes, engineering anthocyanin production in plants does not require engineering the entire pathway, but can be achieved by induction of regulatory proteins of the MBW complex to give high-level accumulation (Figure 4E). Pathway induction by regulatory genes in combination with additional genes encoding decorating enzymes can lead to high level production of novel anthocyanins. TFs also induce the expression of transporters for anthocyanin sequestration in the vacuole [35].

High level production in microbes requires optimization of precursor supply to feed anthocyanin/flavonoid biosynthesis [64]. Engineering the production of highly decorated anthocyanins will require introduction of each gene encoding a decorating enzyme, in addition to the core steps of flavonoid biosynthesis (Figure 1). Engineering anthocyanin production in bacteria has all the same problems asin yeast and, in addition, problems of obtaining full functionality of P450 enzymes and their associated reductases. Anthocyanins may be toxic for microbes, if they have no means of secreting them, so high-level production in microbes may also require engineered secretory systems to ensure they do not accumulate intracellularly. Microbial growth generally requires considerable energy, making it an expensive way to produce anthocyanins on a large scale. Scale-up production for major crops, including vegetables and fruits, is generally easier with lower input requirements and energy

costs. Consequently, it is hard to imagine that production in microbes will ever be as cost-effective as optimized plant production systems.

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**Figure 1**

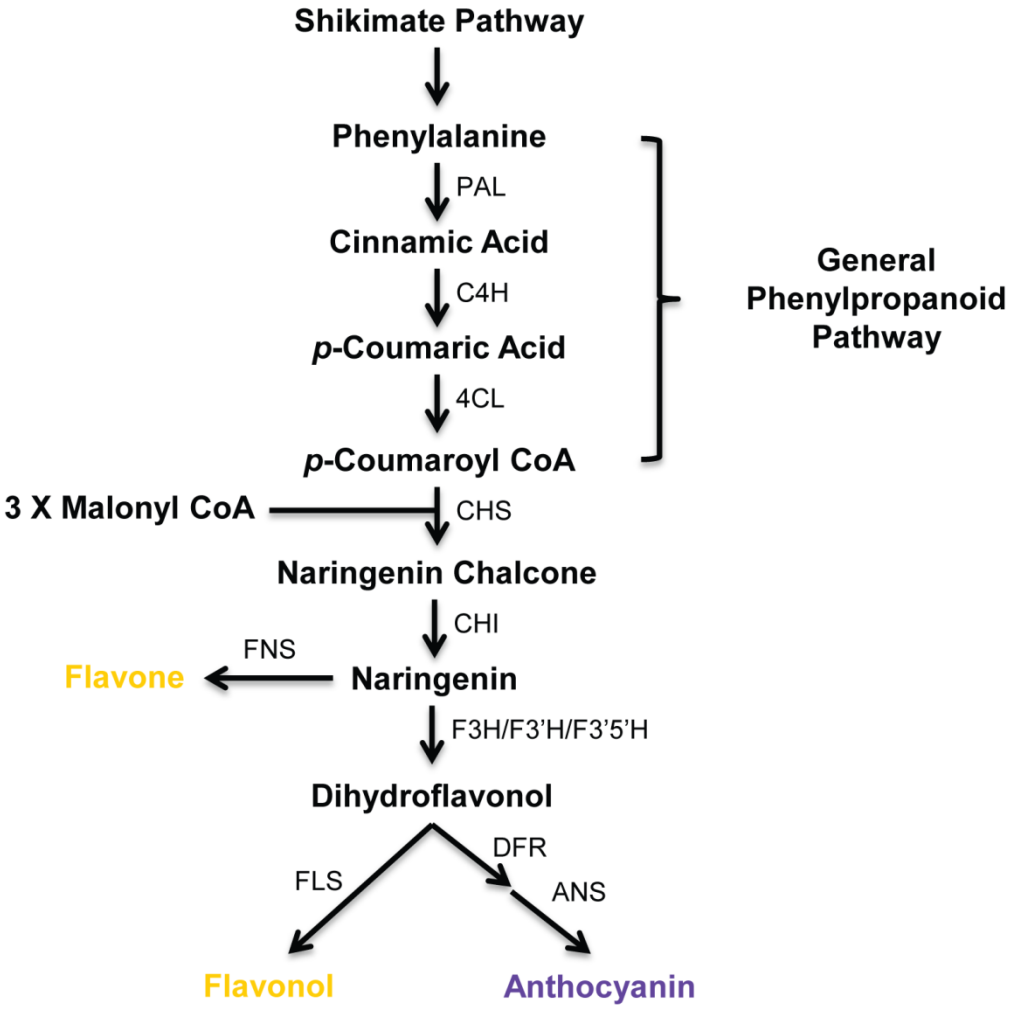
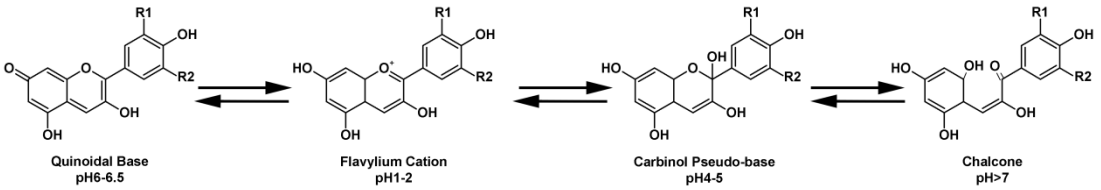



**Figure 1: Core pathway for anthocyanin biosynthesis, showing related pathways for flavonol and flavone synthesis.**

**Figure 2**




Blue Red Colourless Pale yellow

**Figure 2: Changes in conformation of anthocyanins in solution at different pH and the consequent changes in colour.**

**Figure 3**

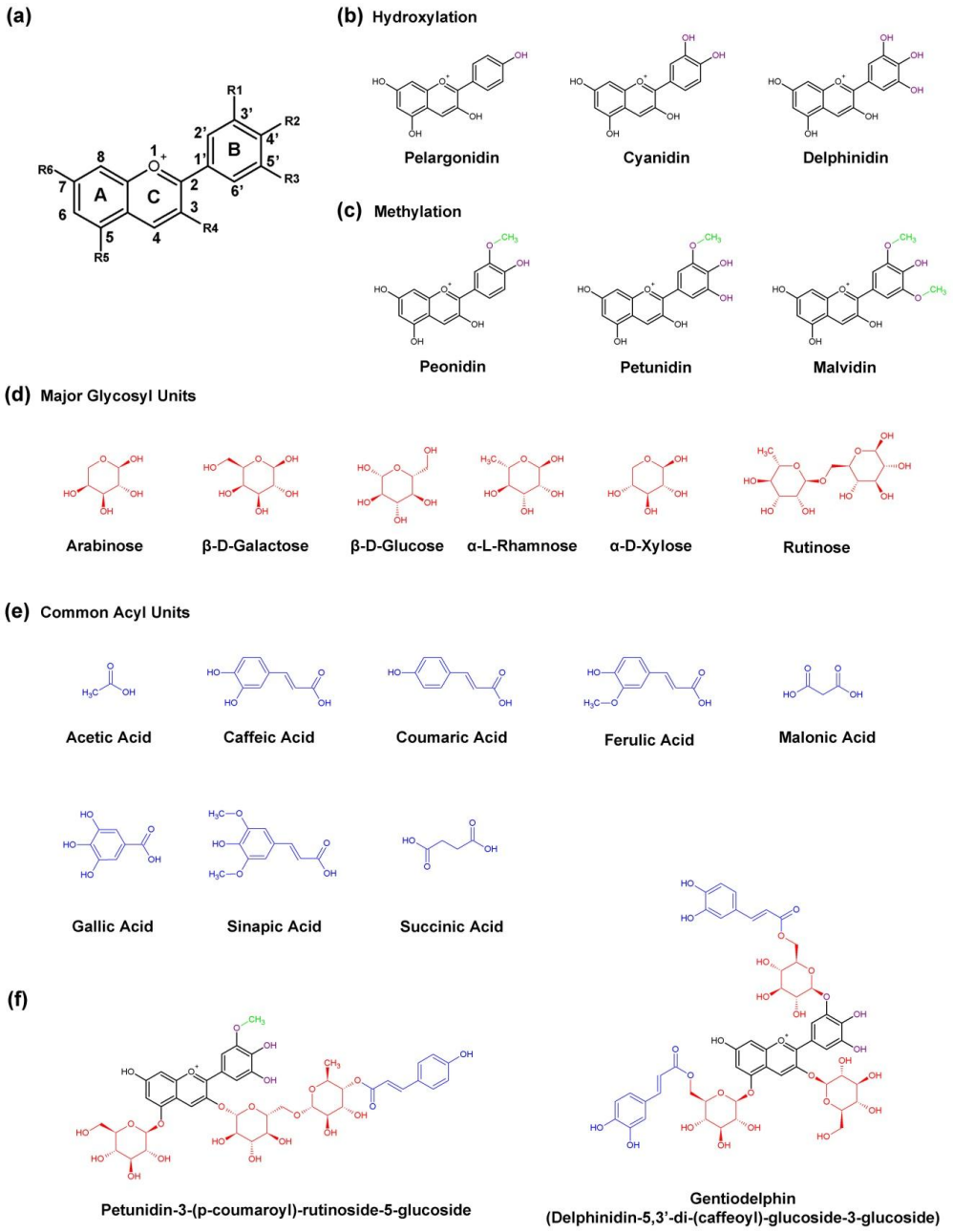



**Figure 3. Decorations of anthocyanins found in nature.** (a) Basic structure of an anthocyanidin showing the numbering of the different carbon groups; common modification positions include 3' (R1), 4' (R2), 5' (R3), 3 (R4), 5 (R5) and 7 (R6) of each ring. (b) Major anthocyanins are distinguished by different hydroxylation patterns. (c) Common anthocyanins are distinguished by their methylation patterns. (d) Major glycosyl units involved in anthocyanin decoration. (e) Common acyl units involved in anthocyanin decoration. (f) Examples of two complex anthocyanins conferring blue-purple colour (petunidin 3-(p-coumaroyl) rutinoside-5 glucoside) in petunia and other common Solanaceous plants, and gentiodelphin, conferring blue colour, in gentian.

**Figure 4**


**Figure 4: Examples of plant cells with engineered anthocyanins.** A) Host Chrysanthemum variety 94-765. B) 94-765 expressing Campanula F3'5'H under the control of the F3H promoter from Chrysanthemum. C) Host Chrysanthemum variety Taihei D) Taihei expressing Campanula F3'5'H under the control of the F3H promoter from Chrysanthemum. Reproduced with permission from reference [64]. E) Tobacco suspension culture cells producing high levels of cyanidin 3-rutinoside as a result of transformation with genes encoding the transcription factors Rosea1 and Delila under the control of the CaMV 35S promoter. Photograph by Kalyani Kallam. F) Tomatoes of the same age left on the vine. The red tomatoes on the left are from a cross of Money Maker x MicroTom. The purple tomatoes on the right express *Delila* and *Rosea1* under the control of the E8 promoter [35] and are from a cross of Money Maker x MicroTom. The purple tomatoes have longer shelf life than the equivalent red ones due to slower over-ripening and reduced susceptibility to *Botrytis cinerea* [82].