

Achievements and Perspectives in Biochemistry Concerning Anthocyanin Modification for Blue Flower Coloration

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Genetic engineering of roses and other plants of floricultural importance to give them a truly blue petal color is arguably one of the holy grails of plant biotechnology. Toward this goal, bluish carnations and roses were previously engineered by establishing an exclusive accumulation of delphinidin (Dp)-type anthocyanins in their petals via the heterologous expression of a flavonoid 3',5'-hydroxylase gene. Very recently, purple-blue varieties of chrysanthemums were also genetically engineered via a similar biochemical strategy. Although the floral colors of these transgenic plants still lack a true blue color, the basis for the future molecular breeding of truly blue flowers is via the engineering of anthocyanin pathways. Anthocyanins with multiple aromatic acyl groups (often referred to as polyacylated anthocyanins) in the 3'- or 7-position tend to display a more stable blue color than non-acylated anthocyanins. The 7-polyacylation process during the biosynthesis of purple-blue anthocyanins in delphinium (Delphinium grandiflorum) was found to occur in vacuoles using acyl-glucose as both the glucosyl and acyl donor. Glucosyltransferases and acyltransferases involved in anthocyanin 7-polyacylation in delphinium are vacuolar acyl-glucose-dependent enzymes belonging to the glycoside hydrolase family 1 and serine carboxypeptidae-like protein family, respectively. The 7-polyacylation proceeds through the alternate glucosylation and *p*-hydroxybenzoylation catalyzed by these enzymes. p-Hydroxybenzoyl-glucose serves as the phydroxybenzoyl and glucosyl donor to produce anthocyanins modified with a p-hydroxybenzoyl-glucose concatemer at the 7-position. This novel finding has provided a potential breakthrough for the genetic engineering of truly blue flowers, where polyacylated Dp-type anthocyanins are accumulated exclusively in the petals.

Keywords: Anthocyanin • Acyltransferase • Blue flower coloration • Chrysanthemum • Delphinium • Glycosyltransferase • Polyacylation.

Abbreviations: AAGT, acyl-glucose-dependent anthocyanin glucosyltransferase; AAT, anthocyanin acyltransferase; ANS, anthocyanidin synthase; AT, acyltransferase; BAHD, benzylal-cohol acetyl-, anthocyanin-O-hydroxycinnamoyl-, anthra-nilate-*N*-hydroxycinnamoyl/benzoyl- and deacetylvindoline acetyltransferase; Cy, cyanidin; Cy3G, cyanidin 3-O-glucoside; Cy3G7G, cyanidin 3,7-O-diglucoside; Cy3dmG, cyanidin

3-O-(3",6"'-O-dimalonyl)-glucoside; Cy3mG, cyanidin 3-O-6"-O-malonylglucoside; DcAA5GT, acyl-glucose-dependent anthocyanin 5-GT of Dianthus caryophyllus; DFR, dihydroflavonol 4-reductase; DgAA7GT, acyl-glucose-dependent anthocyanin 7-GT of Delphinium grandiflorum; DgAA7BG-GT, acyl-glucose-dependent anthocyanin GT of Delphinium grandiflorum acting on p-hydroxybenzoyl group of Dp3R7BG; DHK, dihydrokaempferol; DHM, dihydromyricetin; DHQ, dihydroquercetin; Dp, delphinidin; Dp3G, delphinidin 3-Oglucoside; Dp3G5G3'G, delphinidin 3,5,3'-O-triglucoside; 3-O-(3",6"-O-dimalonyl)-glucoside; Dp3dmG. delphinidin Dp3mG, delphinidin 3-O-6"-O-malonylglucoside; Dp3R, delphinidin 3-O-rutinoside; Dp3R7G, delphinidin 3-O-rutinoside 7-O-glucoside; Dp3R7BG, delphinidin 3-O-rutinoside 7-O-(6""-O-p-hydroxybenzoyl)-glucoside; ER, endoplasmic reticulum; F3'H, flavonoid 3'-hydroxylase; F3'5'H, flavonoid 3',5'-hydroxylase; GH1, glycoside hydrolase family 1; GT, glycosyltransferase; Pg, pelargonidin; Pg3G, pelargonidin 3-O-glucoside; pHB, p-hydroxybenzoyl; pHBA, p-hydroxybenzoic acid; pHBG, 1-0*p*-hydroxybenzoyl- β -D-glucose; SCPL, serine carboxypeptidaselike; UAGT, UDP-sugar:anthocyani(di)n glycosyltransferase; UGT, UDP-sugar-dependent glycosyltransferase.

Introduction

Floral color is one of the most important traits of ornamental plants (Tanaka et al. 2008). Among a wide variety of floral colors in nature, blue arises primarily from anthocyanins (Tanaka et al. 2008, Yoshida et al. 2009), which are (acyl)glycosides of anthocyanidins (a class of flavonoids) (see **Supplementary Fig. S1** for the general structure), and specifically from delphinidin glycosides. Blue flowers are unknown for many floricultural crops, including three top-selling cut flowers—the carnation (*Dianthus caryophyllus*), the chrysanthemum [*Chrysanthemum* (*Dendranthema*) × *morifolium*] and the rose (*Rosa hybrida*). Blue varieties of these flowers have long been an aspiration of floricultural breeders (Chandler and Tanaka 2007).

The breeding of novel plant varieties of unknown flower colors by means of traditional approaches (such as hybridization) has been difficult, primarily because of the constraints of the gene pool for a given plant species. In addition, attempts to modify a certain trait of a plant by traditional breeding methods

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have often been associated with undesirable alterations of other important traits of the plant. These constraints can be circumvented by genetic engineering, which has been a promising approach to the modification of flower colors. The first exciting achievement of flower color modification by genetic engineering was reported in 1987, where a *dihydroflavonol reductase* (*DFR*) gene of maize was used to manipulate the anthocyanin biosynthetic pathway of a petunia (Meyer et al. 1987). Since then, combined with the advancement of genetics, biochemistry and molecular biology of flavonoid biosynthesis, flower color modification by genetic engineering has been one of the hallmarks of plant biotechnology.

Engineering a true-blue flower color (such as flowers of Royal Blue color) is arguably one of the holy grails of plant genetic engineering. Toward this goal, a bluish carnation was first engineered in 1996 by modifying the anthocyanin biosynthetic pathway (Tanaka et al. 1998) (**Table 1**). This purple-blue carnation served as the world's first example of a genetically modified floricultural crop to be marketed. A bluish rose was subsequently genetically engineered through a similar approach (Katsumoto et al. 2007) (**Table 1**) and has been commercially available in Japan since 2009 (Tanaka et al. 2009). Very recently, two research groups reported the genetic engineering of purple-blue varieties of chrysanthemums through an approach essentially similar to those employed in carnations and roses (Brugliera et al. 2013, Noda et al. 2013) (**Table 1**).

Recent elucidation of the biochemistry of anthocyanin modification in vacuoles (Matsuba et al. 2010, Nishizaki et al. 2013) is another important achievement relative to blue flower coloration. The blue color of some flowers, such as cineraria (Senecio cruentus), delphinium (Delphinium grandiflorum) and campanula (Campanula medium), arises from anthocyanins decorated with multiple aromatic acyl groups (see Supplementary Fig. S2 for examples of aromatic acyl groups) at the 7-position (Yoshida et al. 2009), and this type of modification (often referred to as polyacylation) is important for a stable blue coloration. In a recent study, 7-polyacylation during anthocyanin biosynthesis in the delphinium was shown to take place in vacuoles using acyl-glucose as a 'zwitter donor' for both glucosyl and acyl groups (Fig. 1) (Nishizaki et al. 2013). This finding points to another potential strategy for a genetically engineered truly blue flower.

This Mini Review deals with the biochemistry of anthocyanin biosynthesis related to blue flower coloration. It highlights recent achievements in engineering a more brilliant blue shade of chrysanthemum flowers as well as the elucidation of the biosynthetic pathways of 7-polyacylated anthocyanins.

Chemical Basis of a Strategy to Engineer Bluish Chrysanthemums, Carnations and Roses

The hue of anthocyanidins—the chromophore of anthocyanins—is affected by the B-ring hydroxylation patterns. An increase in the number of hydroxy groups on the B-ring of the anthocyanidin chromophore results in a bathochromic shift of the color towards blue (Tanaka and Brugliera 2013). Thus, delphinidin (Dp)-type anthocyanins (having a 3',4',5'-trihydroxy Bring) are bluer in color than the anthocyanins derived from either pelargonidin (Pg; having a 4'-monohydroxy B-ring and showing orange, pink or red colors) or cyanidin (Cy; having a 3',4'-dihydroxy B-ring and showing red or magenta) (see Supplementary Fig S1). Actually, blue flowers in nature tend to have Dp-type anthocynins, although there are exceptions (Yoshida et al. 2009, Tanaka and Brugliera 2013). Anthocyanidin shows pH-dependent structural changes in aqueous solution, appearing bluer at a higher pH (i.e. red at acidic pH to purple at weakly acidic and neutral pH) (Yoshida et al. 2009). Moreover, under weakly acidic and neutral conditions, anthocyanidins are very unstable and produce colorless structures; hence, anthocyanidin must be stabilized in order to form anthocyanins by glycosylation and acylation in the petal cells (Luo et al. 2007). The color of anthocyanins varies with pH as well, appearing bluer at higher pH. The color and stability of anthocyanins are also affected by their modifications (Yoshida et al. 2009). The complexation of anthocyanin with metal ions (such as Mg^{2+} , AI^{3+} and Fe^{3+}) and the stacking of anthocyanin with polyphenolics (such as flavone and flavonols, termed copigments) cause a stable and bluer coloration (Goto and Kondo 1991, Yoshida et al. 2009).

These observations in turn provide hints for the strategies for engineering blue flowers via genetic manipulation (Tanaka et al. 2009). First, the exclusive accumulation of Dp-type anthocyanins in the petal cells should provide a basis for a bluer coloration of the flower. Chrysanthemums, carnations and roses lack blue varieties primarily because they all lack Dptype anthocyanins (Tanaka et al. 2005). Specifically, anthocyanins that accumulate in the petals (ray florets) of natural chrysanthemum flowers are of a Cy type (Nakayama et al. 1997). In the bluish petals of transgenic chrysanthemum flowers that had been recently engineered by two groups (Brugliera et al. 2013, Noda et al. 2013), these were exclusively replaced by a Dp type. Secondly, other conditions (e.g. high vacuolar pH and the coexistence of co-pigments) might also be needed for a bluer flower. In this regard, there are some cultivars of the chrysanthemum and the carnation that satisfy some of these conditions, thereby favoring a bluer coloration (Tanaka et al. 2009). Roses, however, do not fulfill any of these conditions completely (Katsumoto et al. 2007).

Biochemistry of the Exclusive Accumulation of Dp-Type Anthocyanins

The biosynthetic pathway of anthocyanins from phenylalanine via chalcone to anthocyanidins (**Fig. 2**) is well conserved among seed plants (Winkel-Shirley 2001, Davies and Schwinn 2005, Tanaka et al. 2008). In many plant species (including carnations and chrysanthemums), the resultant anthocyanidins subsequently undergo 3-O-glucosylation catalyzed by anthocyanidin 3-O-glucosyltransferase (EC 2.4.1.115; UA3GT, see below). This is followed by further enzymatic modifications (such as O-methylation, glycosylation and acylation) to produce a great

Strategy and results	Carnation (Dianthus ca	ryophilus)	Rose (Rosa $ imes$ hybrida)	Chrysanthemum (Chrysanthemum × morifoliu	m)
	cv. Moondust (Tanaka et al. 1998, Fu 2003, Tanaka et al. 200	cv. Moonshadow Ikui et al. 39)	cv. Applause (Tanaka et al. 2009, Katsumoto et al. 2007)	(Noda et al. 2013)	(Brugliera et al. 2013)
Introduction Plant source of F3/5/H of F3/5/H	Petunia (<i>Petunia</i> × hybrida)	Pansy (Viola $ imes$ wittrockiana)	Pansy (Viola × wittrockiana)	Campanula (Campanula medium)	Pansy (Viola $ imes$ wittrockiana)
Promoter (p) used to drive F3 [/] 5 [/] H	Snapdragon CHSp	Snapdragon CHS <i>p</i>	Enhanced CaMV 35Sp	Chrysanthemum F3Hp	Rose CHSp
Eliminating endogenous activities of F3'H and DFR	DFR and F3′H mutant	DFR mutant	NDa	QN	The introduced F3'5'H partly outcompeted the endogenous DFR activity. Rose CHSp-driven RNAi of F3'H
Enhancing the Plant source of DFR Dp pathway Promoter (p) used to drive DFR	Petunia × hybrida Mac1p	Petunia × hybrida Its own p	ND^{a}	ND	DN
Choice of a host cultivar with an appropriate native background for bluish coloration	A strong co-pigment eff apigenin glycosides, a vacuolar pH of 5.5.	ect of the co-existing relatively high	(i) Visual selection of pink and mauve roses, followed by (ii) flavonoid analysis and pH measurement of their petal extracts, to find cultivars with a high vacuolar pH, large amounts of flavonols and less F3'H activity	Cultivars (Taihei and 9-765) that were most suitable for transformation were selected as hosts ^b	Host cultivars with a suitable background were screened by substrate feeding assays
The highest Dp level in total anthoccyanidin	100%	94%	95%	92–95%	80%
RHSCC number of petal color	75A (mauve)	82A (purple)	85B (violet)	83B and 88C (violet)	e.g. N81B (purple-violet)
Structures of major anthocyanins	Delphinidin 3,5-O-digluco diester, delphinidin 3,5 and delphinidin 3,5-O- 6″,6″-O-dimalyl ester	oside 6″0-4,6″''-0-cyclic-malyl 5-0-diglucoside 6″-0-malyl ester, -diglucoside	3-O-Glucoside (10%) and 3,5-O-diglucoside (90%) of delphinidin	3-0-6″-0-Malonyl- and 3-0-(3″,6″-0-dimalonyl)- glucosides of delphinidin	NR
Co-pigments that accumulate in the petal	Kaempferol 3-(6'''-rham kaempferol 3-(6'''-rhar glucosyl)-glucoside), ar glucoside-6'''-malyl est	nosyl-2'''-glucosyl-glucoside), mnosyl-2'''-(6-malyl- nd apigenin 6-C-glucosyl-7-0- :er	Myricetin, quercetin and kaempferol types of flavonols with a myricetin type being major flavonol	Apigenin and luteolin types of flavones	Apigenin, luteolin and tricetin types of flavones
Abbreviations: CHS. chalcone synthase: Ca	MV. Cauliflower mosaic virus;	DFR. dihvdroflavonol 4-reductase; F3H,	flavanone 3-hydroxylase; F3'H, flavonoid 3'-hyd	troxvlase: E3'5'H. flavonoid 3'.5'-hvd	roxvlase: RNAi. RNA interference: p.

PHYSIOLOGY

promoter; ND, not done; NR, not reported.

^a The transgenic lines with the expressed pansy F3'5'H showed a Dp level of 95% without manipulating DFR and were selected for commercial release (Dr. Yoshikazu Tanaka, Suntory Holdings, Japan; personal communication). ^b Dr. Naonobu Noda, NARO Institute of Floricultural Sciences, Japan; personal communication





Fig. 1 Proposed biosynthetic pathways of viodelphin and cyanodelphin in the delphinium.

structural diversity depending on plant species and varieties. It is believed that anthocyanin biosynthesis takes place in the cytoplasm (Tanaka et al. 2008). Moreover, there are several lines of evidence for metabolon formation. In the metabolon, the flavonoid biosynthetic enzymes are weakly associated with each other and form multienzyme complexes on the cytoplasmic surface of the endoplasmic reticulum (ER) (Stafford 1990, Burbulis and Winkel-Shirley 1999, Winkel 2004). In petal cells, anthocyanins thus produced ultimately accumulate in vacuoles (Tanaka et al. 2008). However, recent clarification has shown that enzymatic modification of anthocyanins can also take place in the vacuoles (see below for details).

The B-ring hydroxylation patterns of flavonoids are primarily governed by the occurrence of the expressed flavonoid 3'hydroxylase (F3'H; EC 1.14.13.21) and flavonoid 3',5'-hydroxylase (F3'5'H; EC 1.14.13.88) enzymes in the cell (see **Fig. 2**) (Tanaka and Brugliera 2013). F3'H and F3'5'H modify the Bring of flavonoids (flavanones, dihydroflavonols, flavones and flavonols; see **Fig. 2** for structures) to produce 3',4'-dihydroxy and 3',4',5'-trihydroxy structures, respectively (Tanaka and Brugliera 2013). Reaction of dihydrokaempferol (DHK; one hydroxy group on the B-ring) with F3'H and F3'5'H yields dihydroquercetin (DHQ; two hydroxy groups) and dihydromyricetin (DHM; three hydroxy groups), respectively (see Fig. 2). The absence of these enzymes in the petal cell results in the production of flavonoids with a 4'-monohydroxy B-ring structure. The absence of Dp-type anthocyanins in the petals of the carnation, the chrysanthemum and the rose is primarily due to the absence of the F3'5'H gene in their genomes (Tanaka et al. 2009, Tanaka and Brugliera 2013). F3'H and F3'5'H are ER-localized, heme-containing Cyt P450s (designated CYP75B and CYP75A, respectively, according to Cyt P450 nomenclature) and show a close evolutionary relationship with each other (Tanaka and Brugliera 2013). The differentiation of CYP75B and CYP75A is believed to have occurred before the speciation of flowering plants (Seitz et al. 2006, Ishiguro et al. 2012). The angiosperms originally produced purple-blue flowers containing Dp-type anthocyanins. During the course of angiosperm evolution, it is believed that flower color variations were produced from blue to red through the loss of the F3'5'H function (Rausher 2008). In some Asteraceae plants producing Dp (e.g. aster and cineraria), however, the reversal (color variations from red to blue) also took place after the loss of the CYP75A-related F3'5'H, where F3'5'H evolved from a CYP75Brelated ancestor (Seitz et al. 2006) following an amino acid substitution (threonine/serine). F3'5'H serves as a key player in the engineering of bluish flowers by genetic manipulation. F3'H also plays an important role in flower color engineering.





Fig. 2 Biosynthetic pathways of anthocyanins and related flavonoids. Names of flavonoids are shown under their chemical structures. Names of flavonoid classes are boxed. Abbreviations of enzyme names are: PAL, phenylalanine ammonia lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate:CoA ligase; CHS, chalcone synthase; Ch4'GT, chalcone 4'-glucosyltransferase; AS, aureusidin synthase; CHI, chalcone isomerase; IFS, 2-hydroxyisoflavanone synthase; HID, 2-hydroxyisoflavanone dehydratase; FNS, flavone synthase; F3H, flavanone 3-hydroxylase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; UA3GT, anthocyanidin 3-glucosyltransferase; FLS, flavonol synthase; F3'H, flavonoid 3'-hydroxylase; F3'F, flavonoid 3'5'-hydroxylase. Note that F3'H can also utilize naringenin and F3'5'H can utilize eriodyctyol and dihydroquercetin as substrates, though they are not shown in this figure. Cy-, Pg- and Dp-type anthocyanins generally show crimson-red, orange-pink and violet-blue color, respectively, as shown in the background, although there are exceptions. The yellow petal color that arises from chalcones, aurones and flavonols is typified in cosmos (*Cosmos bipinnatus*), snapdragon (*Antirrhinum majus*) and camellia (*Camellia chrysantha*) (Tanikawa et al. 2008), respectively.

Dihydroflavonols (DHK, DHQ and DHM) thus produced undergo a stereo-specific reduction at their 4-position that is catalyzed by dihydroflavonol 4-reductase (DFR; an NADPH-dependent oxidoreductase; EC 1.1.1.219) to produce colorless leucoanthocyanidins (flavan-3,4-diols). Leucoanthocyanidins are converted to anthocyanidins (the first colored compounds in the biosynthetic pathway) via the action of anthocyanidin synthase [ANS; a member of the 2-oxoglutarate-dependent



dioxygenase family (Kawai et al. 2014); EC 1.14.11.19]. DHK, DHQ and DHM each undergoes successive DFR- and ANS-catalyzed reactions in a parallel manner in the petal cell (see **Fig. 2**) (Davies and Schwinn 2005).

Substrate specificities of DFR vary with plant species (Beld et al. 1989, Martens et al. 2002) and are known to exert a strong impact on the B-ring hydroxylation pattern of anthocyanins that accumulate in the petal cell (Meyer et al. 1987, Beld et al. 1989, Johnson et al. 1999). Specifically, whereas DFRs from many plants, including carnations, chrysanthemums and roses, accept all types of dihydroflavonols (DHK, DHQ and DHM), the DFRs of petunias, cymbidiums and irises act on DHQ and DHM but not on DHK (Supplementary Table S1). The absence of the Pg-type anthocyanins in petunias, cymbidiums and irises is in part explained in terms of the substrate specificity of the DFR of these plant species. Thus far, attempts have been made to identify a determinant of substrate specificity in DFR. On the basis of sequence comparison, Beld et al. (1989) first predicted that a stretch of 13 amino acids of DFR might be responsible for substrate specificity. Subsequently, Johnson et al. (2001) were able to alter the substrate specificity of Gerbera DFR by changing a single amino acid residue (Asn134 \rightarrow Leu) located in a 26 amino acid region that overlaps with the 13 amino acid region. In 2007, the crystal structure of the grape (Vitis vinifera) DFR complexed with NADP and DHQ was determined (Petit et al. 2007). According to the stereo structure, a 26 amino acid segment ranging from position 131 to 156 of the DFR of the grape probably controls the recognition of the B-ring hydroxylation pattern of the substrate dihydroflavonols (Petit et al. 2007).

The biosynthetic pathway of flavonoids has been generally described in terms of a metabolic grid (Stafford 1990, Weng and Noel 2012), where different enzymes compete for the same substrate and the same enzyme can act on different substrates. As such, the heterologously expressed F3'5'H in the petal cells of transgenic plants potentially competes with the endogenous DFR for DHK and also competes with endogenous F3'H for naringenin and DHK (Fig. 2). Such competition will hamper the exclusive accumulation of Dp-type anthocyanins in the petals of the transgenic flowers. Thus, to make petals as blue as possible on the basis of the exclusive accumulation of Dptype anthocyanins in the petal cells by means of the heterologous expression of F3'5'H, the following points must also be considered (Tanaka et al. 2009): (i) the formation of Pg and Cy must be minimized by eliminating the endogenous activities of F3'H and DFR, which are capable of acting on DHK; (ii) the Dp pathway could be enhanced by expressing a DFR with specificity in favor of Dp formation (such as DFR from petunia) as required; and, finally, (iii) to make petals as blue as possible, the choice of a host cultivar with an appropriate native background (such as higher vacuolar pH, higher concentrations of co-pigments, etc.) would also be important.

Previous successful engineering of bluish carnations (Tanaka et al. 1998, Fukui et al. 2003) and roses (Katsumoto et al. 2007) has been accomplished taking all of these aspects into consideration, as summarized in **Table 1**. It must also be mentioned that modification patterns of Dp-type anthocyanins that

accumulate in the bluish petals of the transgenic carnations (Fukui et al. 2003) and roses (Katsumoto et al. 2007) that have been obtained thus far were analogous to those of the anthocyanins in the red petals of the corresponding natural plants (**Table 1**). Thus, the anthocyanin-modifying enzymes of these plants that are positioned downstream of anthocyanidin in the biosynthetic pathway (**Fig. 2**) could also act on the Dp types.

Engineering of Bluish Chrysanthemum Flowers Based on Dp Accumulation

In the chrysanthemum, the cyanidin 3-O-glucoside (Cy3G) that is produced (as shown in **Fig. 2**) is further malonylated at its 6"- and 3"-positions by the action(s) of malonyl-CoA:anthocyanin malonyltransferases Dm3MaT1 and/or Dm3MaT2 (EC 2.3.1.171; **Supplementary Fig. S3**) (Suzuki et al. 2004a, Unno et al. 2007). Thus, the major anthocyanins that accumulate in the petals (ray florets) of chrysanthemums are 3-O-6"-O-malonyl- and 3-O-(3",6"-O-dimalonyl)-glucosides of Cy (termed Cy3mG and Cy3dmG, respectively) (Nakayama et al. 1997). Besides the absence of Dp-type anthocyanins, chrysanthemums also lack the Pg-type, primarily due to the strong F3'H activity that outcompetes DFR for DHK (Schwinn et al 1994).

Attempts were made to express F3'5'H cDNAs and other cDNAs from several plant sources in chrysanthemums heterologously in order to engineer a blue flower. However, in these cases, the accumulation of Dp-type anthocyanins in the petals of the transgenic chrysanthemum failed (see He et al. 2013, for example), probably because of the instability in the transgene expression as well as other unexpected effects in the transgenic chrysanthemum plant (Noda et al. 2013).

In the work of Brugliera et al. (2013), pink cultivars of daisytype chrysanthemums, which showed low Cy and high flavone (a co-pigment) content, were selected and the petals visually assessed for accumulation of bluish hues after being fed DHM. This allowed the selection of host cultivars with a background that is expected to ensure a bluish coloration of the petal after the transformation with F3'5'H and other necessary genes. When the selected cultivars were transformed with a pansy F3'5'H under the control of a rose CHS (chalcone synthase) promoter fragment, one of the resultant transgenic lines accumulated Dp at a level of 40% of the total anthocyanin in flower petals. Although this transgenic line contains endogenous DFR activity capable of acting on DHK (see above), the heterologous F3'5'H appeared to outperform this DFR activity effectively. The Dp levels in the petals of transgenic flowers were further enhanced to 80% by hairpin RNA interference (hpRNAi)mediated silencing of the endogenous F3'H. Flavonoid analysis also revealed the accumulation of tricetin (5,7,3',4',5'-pentahydroxyflavone) in the petals of the transgenic chrysanthemum, in addition to apigenin (5,7,4'-trihydroxyflavone) and luteolin (3,7,3',4'-tetrahydroxyflavone). Thus, in the transgenic chrysanthemum, B-ring hydroxylation patterns of flavones were also affected by the expression of the pansy F3'5'H gene,

as in the case of the transgenic rose expressing pansy F3'5'H where myricetin (5,7,3',4',5'-pentahydroxyflavonol) accumulated as a major flavonol.

In the work of Noda et al. (2013), the most suitable promoters for Dp production in ray florets were screened using a pansy F3'5'H cDNA as a transgene. The petal-specific chrysanthemum flavanone 3-hydroxylase promoter (CmF3Hp) gave the highest level of Dp accumulation. These authors then examined F3'5'H genes of various plant origins to find the best candidate for expression under the control of CmF3Hp and found that the use of the F3'5'H from campanula gave rise to the highest delphinidin content in total anthocyanidins. They also examined the use of translational enhancers functioning in the chrysanthemum in an attempt to improve campanula F3'5'Hexpression. Taken together, the highest level of Dp accumulation (92-95% of total anthocyanidins) was obtained. HPLC analysis showed that anthocyanins accumulating in the transgenic flowers were 3-0-6"-O-malonyl- and 3-0-(3",6"-Odimalonyl)-glucosides of Dp (i.e. Dp3mG and Dp3dmG, respectively). Their modification patterns were analogous to those of the Cy-type anthocyanins found in the red petals of natural plants, consistent with the previous observation that Dm3MaT1 and Dm3MaT2 were capable of efficiently malonylating Dp3G (Supplementary Fig. S3) (Suzuki et al. 2004a). Flavonoid analysis also revealed that luteolin- and apigenintype flavones accumulated exclusively in the ray florets of the transgenic chrysanthemum and that tricetin was essentially absent (Noda et al. 2013). This observation was unlike the case reported by Brugliera et al. (2013), where tricetin was accumulated (see above). The absence of tricetin in the ray florets of the transgenic chrysanthemum may be in part explained in terms of differences in the spatial and/or temporal expression of flavone biosynthetic genes and F3'5'H.

Purple-blue varieties of lilies (Lilium) (Tanaka et al. 2012), phalaenopsis (Phalaenopsis amabilis) (Suzuki et al. 2008) and dahlia $(Dahlia \times hybrida)$ (Dr. Masahiro Mii, Chiba University, personal communication) were also genetically engineered through a similar strategy. The present success in engineering a bluish chrysanthemum has further corroborated the general feasibility of the approach to engineer bluish flowers through the accumulation of Dp-type anthocyanins in the petals. It was previously pointed out that the flowers engineered through this approach lacked a true Royal Blue hue (Anonymous 2009). However, because blue flowers in nature tend to have Dp-type anthocynins (see above), this approach should provide an important basis for the future engineering of such truly blue flowers. Genetic manipulation of flowers that causes an accumulation of the polyacylated Dp-type anthocyanins (see below) in their petal cells could be one of these promising strategies.

Polyacylated Anthocyanins and Their Stable Blue Coloration

In many plant species, anthocyanins are modified with aromatic acyl groups [e.g. *p*-coumaroyl, caffeoyl and feruloyl, sinapoyl, *p*-hydroxybenzoyl (pHB) and vanillyl groups] and/or aliphatic acyl

groups (e.g. acetyl, malonyl, succinyl and malyl groups) in their structures (**Supplementary Fig. S2**). These acyl groups are commonly linked to the hydroxy groups of the glycosyl moieties of anthocyanins. Anthocyanins with multiple aromatic acyl groups are often referred to as 'polyacylated' anthocyanins and tend to display a stable blue color. More than 100 types of polyacylated anthocyanins have been identified (see **Supplementary Fig. S4** for examples) (Honda and Saito 2002). The stable blue coloration of these polyacylated anthocyanins is believed to arise mainly from the face-to-face intramolecular stacking of the aromatic groups with the chromophore anthocyanidin (Goto and Kondo 1991, Honda and Saito 2002, Yoshida et al. 2009).

Thus far, the aromatic acyl groups in the B-ring (i.e. in the 3'position of the anthocyanin) are known to stack effectively with the chromophore to give rise to the bluish coloration (Yoshida et al. 2000, Yoshida et al. 2009). The aromatic acyl groups in the 7-position of the anthocyanin are also important for bluish coloration. The 7-aromatic acylations show cumulative bathochromic shifts of the UV/VIS absorption maximum (a 10 nm shift per aromatic acyl group) even under acidic conditions (Nishizaki et al. 2013). The anthocyanins modified with aromatic acyl groups in their 3- and/or 5-positions appear to provide only a reddish-purple color (without the interaction with metal ions and/or co-pigments) even though their chromophore is Dp (Shiono et al. 2005, Takeda et al. 2005, Shoji et al. 2007, Yoshida and Negishi 2013). It is therefore believed that 3'- and/or 7-aromatic acylations are more important than 3- and/or 5-aromatic acylations for the blue coloration of anthocyanins. The gentiodelphin of gentians (Gentiana triflora) and the ternatins of butterfly peas (Clitoria ternatea) are examples of bluish anthocyanins with 3'-polyacylation. The viodelphin and cyanodelphin of delphinium, the cinerarin of cineraria and the campanin of campanula are examples of bluish anthocyanins with 7-polyacylation (Supplementary Fig. S4). In structural terms, it is noteworthy that many of these bluish polyacylated anthocyanins have concatenate acyl-glucose units in their structures (e.g. ternatin A1, viodelphin, cyanodelphin, cinerarin and campanin), although there are exceptions (e.g. gentiodelphin and lobelinin B) (Honda and Saito 2002).

Glycosyltransferases and Acyltransferases Involved in Anthocyanin Modification

Anthocyanin modification with glycosyl and acyl groups (including aromatic acyl groups) is catalyzed by glycosyltransferases (GTs) and acyltransferases (ATs), respectively, in an appropriate order, where the glycosylation by GTs provides the basis of the acylation by ATs. Glycosylation and acylation of anthocyanins play important roles in maintaining their color stability and are believed to serve as signals for vacuolar transport (Luo et al. 2007). Evolutionarily distinct types of GTs and ATs are involved in plant secondary metabolism (including anthocyanin biosynthesis), and this illustrates the convergent evolution of different protein families to acquire a similar biological function (Weng et al. 2012).



Anthocyanin GTs

To date, two evolutionarily distant groups of anthocyani(di)n GTs have been identified. One of these groups is a member of the UDP-sugar-dependent GT family 1 (UGT) (Yonekura-Sakakibara and Hanada 2011). The family 1 UGTs generally catalyze glycosylation of small molecules and represents the largest GT family in plants-for example, 115 and 213 UGT genes have been found in Arabidopsis thaliana and Oryza sativa genomes, respectively (Yonekura-Sakakibara and Hanada 2011). A large number of anthocyani(di)n GTs (UAGTs) belonging to this family have been identified (Supplementary Table S2). UAGTs catalyze the transfer of a glycosyl group from UDP-sugar to a specific position (the 3-, 5or 3'-position) of the glycosyl acceptor anthocyani(di)n (herein referred to as UA3GT, UA5GT and UA3'GT, respectively; see Supplementary Fig. S5 for some examples); UAGTs that are specific for the 7-position of anthocyani(di)ns have not yet been identified. The UA3'GTs provide the basis of 3'-polyacylations, and, thus far, two such enzymes have been identified: GtUA3'GlcT of G. triflora, which is involved in gentiodelphin biosynthesis (Fukuchi-Mizutani et al. 2003), and anthocyanin 3',5'-glucosyltransferase (CtUA3'5'GlcT) of C. ternatea (Kogawa et al. 2007), which catalyzes 3'- and 5'-glucosylation of Dp3mG and is involved in ternatin biosynthesis (Supplementary Fig. \$5). Many of the UAGTs reported thus far are glucosyltransferases, but the sugar donor specificity of UAGTs may vary with the source of the enzyme, which also includes galactosyltransferases (Montefiori et al. 2011), glucuronosyltransferases (Sawada et al. 2005), rhamnosyltransferases (Kroon et al. 1994) and xylosyltransferases (Montefiori et al. 2011, Yonekura-Sakakibara et al. 2012). Many UGTs are thought to be cytoplasmic enzymes (Lim and Bowles 2004), consistently showing an optimum pH for catalytic activities in the neutral to slightly alkaline region. For comparison, most vacuolar enzymes show an optimal pH for their activities that is in the acidic to slightly acidic region (i.e. the pH of the vacuole lumen) (Boller and Kende 1979).

Phylogenetic analysis has revealed that the positional specificities of the glycosyl transfer to flavonoids (including anthocyanins) that is catalyzed by UGTs were closely related to the phylogenetic clusters of the enzymes (Vogt and Jones 2000, Noguchi et al. 2009, Ono et al. 2010, Yonekura-Sakakibara and Hanada 2011) (see Supplementary Fig. S6), where cluster I (specific for the 3-position of flavonoids), cluster II (specific for the 5-position), clusters IIIa and IIIb [specific for the 7-position of flavonoids other than anthocyanins] and cluster IV (specific for the glycon moiety) have been identified. Cluster I exceptionally includes CtUA3'5'GlcT (Supplementary Fig. S5). Clusters III do not include anthocyanin 7-O-glycosyltransferase, but they do include anthocyanin 5,3-glucosyltransferase (RhUA53GlcT) of the rose (cluster IIIb) (Ogata et al. 2005), 3-deoxyanthocyanidin 5-O-glucosyltransferase (ScUdA5GlcT/ ScUGT5) of Sinningia cardinalis (cluster IIIb) (Nakatsuka and Nishihara 2010) and GtUA3'GlcT (cluster IIIa) (Fukuchi-Mizutani et al. 2003) (Supplementary Figs. S5, S6). The analysis also revealed that the sugar donor specificity of UGTs could

be differentiated within each phylogenetic cluster after establishment of the positional specificity of the sugar acceptor in specific plant lineages (Noguchi et al. 2009, Ono et al. 2010, Yonekura-Sakakibara and Hanada 2011).

The acyl-glucose-dependent anthocyanin glucosyltransferases represent the other type of anthocyanin GTs (**Supplementary Table S3**) and were recently identified for the first time in carnations and delphiniums (Matsuba et al. 2010). This type of GT utilizes acyl-glucoses (i.e. 1-*O*- β -*D*-glucose esters of organic acids) as its glucosyl donor [such as 1-*O*-hydroxycinnamoyl- β -*D*-glucoses (e.g. *p*-coumaroyl-, caffeoyl-, sinapoyl- and feruloyl-glucoses) and 1-*O*-hydroxybenzoyl- β -*D*-glucose (e.g. vanillyl- and *p*-hydroxybenzoyl-glucoses)]. Acyl-glucose can serve as a high-energy donor molecule with a high group transfer potential in acylglucose-dependent transfer reactions; the standard Gibbs' free energy change of the hydrolysis of sinapoyl-glucose was previously estimated to be -35.7 kJ mol⁻¹ (Mock and Strack 1993).

The carnation and delphinium enzymes catalyze the 5- and 7-O-glucosylation of anthocyanidin 3-O-glucosides [termed DcAA5GT (acyl-glucose-dependent anthocyanin 5-GT) and DgAA7GT, respectively] (Matsuba et al. 2010) (Supplementary Fig. S7). These GTs were shown to occur in non-cytoplasmic spaces (vacuoles) in the petal cell (Matsuba et al. 2010). Consistently, glucose conjugates of organic acids (including acyl-glucoses) are known to accumulate in vacuoles (Yazaki et al. 1995, Eudes et al. 2008, Luang et al. 2013), and the native DcAA5GT purified from carnation petals displays an optimum pH for its catalytic activity at around pH 4.5-5.0 (Matsuba et al. 2010). DgAA7GT was the first example of an enzyme that was capable of specifically glucosylating the 7-position of anthocyanin and serves as the first committed enzyme of 7-polyacylation in the viodelphin/cyanodelphin biosynthesis in this plant (Fig. 1, see also below). AA7GT was also identified in a monocot plant (Agapanthus africanus) (Miyahara et al. 2012). In 2013, two other acyl-glucose-dependent GTs were identified—in Arabidopsis (AtBGLU10) (Miyahara et al. 2013) and delphinium (DgAA7BG-GTs) (Nishizaki et al. 2013). DgAA7BG-GTs catalyze the glucosyl transfer from p-hydroxybenzoyl-glucose (pHBG) to the phenolic hydroxy group of the pHB moieties of 7-glucosylated anthocyanin (Nishizaki et al. 2013) (Supplementary Fig. S7). The DgAA7BG-GTs were shown to be vacuolar proteins (Nishizaki et al. 2013) and should be involved in elongation of the concatenate acyl-glucose units in the 7-position of anthocyanin during the biosynthesis of viodelphin/cyanodelphin (Fig. 1).

It is significant that all these acyl-glucose-dependent GTs were found to be members of the glycoside hydrolase family 1 (GH1) (Matsuba et al. 2010, Nishizaki et al. 2013) and thus are collectively referred to here as GH1-GTs. Thus far, biological functions of GH1-family enzymes have mainly been described in terms of their hydrolytic activities. Generally, however, gly-cosidases are also capable of catalyzing transglycosylation, where the glycosyl group is transferred to nucleophiles (e.g. alcohols) other than water, and the abilities of glycosidases to catalyze transglycosylation may vary with the enzyme sources. DgAA7GT consistently displays weak hydrolytic activities on



their substrates (k_{cat}/K_m for pHBG, $53 \text{ s}^{-1} \text{ M}^{-1}$; k_{cat}/K_m for Cy3G5G, $170 \text{ s}^{-1} \text{ M}^{-1}$) and products (k_{cat}/K_m for Cy3G7G, $190 \text{ s}^{-1} \text{ M}^{-1}$) in the absence of a glucosyl donor and acceptor. However, these values are much lower than those of glucosyl transfer activities in the presence of a glucosyl donor and acceptor (k_{cat}/K_m for pHBG, $580 \text{ s}^{-1} \text{ M}^{-1}$; k_{cat}/K_m for Cy3G, $7,300 \text{ s}^{-1} \text{ M}^{-1}$) (Matsuba et al. 2010). Indeed, the incubation of pHBG and Cy3G with the enzyme results in exclusive production of the transfer product Cy3G7G (not Cy) (Matsuba et al. 2010).

Phylogenetic analysis has suggested that GH1-GTs might evolve from a hydrolase member of the GH1 family (Nishizaki et al. 2013). GH1-GTs acting on anthocyanins identified thus far do not necessarily belong to the same phylogenetic clade. For example, in the delphinium, AA7BG-GTs might not be generated by the gene duplication of an ancestor gene common to AA7GT. Moreover, AtBGLU10 and DgAA7BG-GTs both show a similar specificity (catalyzing the glucosyl transfer to the hydroxy group of an aromatic acyl group of anthocyanins), but are located in different clades (Miyahara et al. 2013, Nishizaki et al. 2013). These observations imply that the conversion of a glycoside hydrolase to a glycosyltransferase might easily occur during enzyme evolution. Phylogenetic analysis has also suggested that many other plant species are also likely to have GH1-GTs, but the acceptor specificities remain to be clarified. It also is noteworthy that the GH1-GTs identified thus far have been exclusively glucosyltransferases. At present, GH1-GTs that transfer glycosy groups other than glucose remain to be identified, although the GH1 family includes glycosidases of other sugar specificities such as β -galactosidases and β -mannosidases (see fig. 8 of Nishizaki et al. 2013).

AATs

Two evolutionarily distinct types of anthocyanin acyltransferases (AATs) have been identified. One catalyzes the position-specific transfer of acyl groups to the sugar moiety of anthocyanins using acyl-CoA as the donor (Nakayama et al. 2003). These enzymes (Supplementary Table S4) are members of the BAHD family, which was named according to the first letter of each of the first four biochemically characterized members (benzylalcohol acetvltransferase, anthocyanin-O- hydroxycinnamoyltransferase, anthranilate-N-hydroxycinnamoyl/benzoyltransferase and deacetylvindoline acetyltransferase). The BAHD family is a large protein family consisting of acyl-CoA-dependent acyltransferases involved in secondary metabolism in plants and fungi (D'Auria 2006). The genomes of A. thaliana and O. sativa have been estimated to encode 64 and 119 genes of BAHD members, respectively (D'Auria 2006). The BAHD members have been thought to evolve rapidly, and their specificities and biochemical roles in plants are very versatile (Nakayama et al. 2003, D'Auria 2006, Luo et al. 2007). AATs belonging to this family, referred to as BAHD-AATs, can be strictly classified on the basis of acyldonor specificity into aliphatic and aromatic BAHD-AATs (Nakayama et al. 2003). Dm3MaT1 and Dm3MaT2 of the chrysanthemum (Suzuki et al. 2004a, see above) are examples of aliphatic BAHD-AATs (Supplementary Fig. S3). An aromatic

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BAHD-AAT that is responsible for the 3'-acylation of anthocyanin (Gt53'AT) was isolated from gentian, which transfers hydroxycinnamoyl groups from hydroxycinnamoyl-CoA to both the 5- and 3'-glucosyl moieties of Dp3G5G3'G (Fujiwara et al. 1997, Fujiwara et al. 1998, Mizutani et al. 2006) (**Supplementary Fig. S3**). BAHD-AATs are believed to be cytoplasmic enzymes (Fujiwara et al. 1998), and their pH optimum is generally in the neutral to slightly alkaline range.

BAHD-AATs often share a unique sequence, -Tyr-Phe-Gly-Asn-Cys-, in addition to the His-Xaa-Xaa-Xaa-Asp- and the -Asp-Phe-Gly-Trp-Gly- motifs conserved among all BAHD family members. Phylogenetic analysis has consistently shown that BAHD-AATs group together with other BAHD members involved in the modification of other flavonoids and phenolic glucosides into a single cluster (cluster I) that is separated from other BAHD family members (see Supplementary Fig. S8). The analysis also suggests that BAHD-AATs may be further subclustered based on plant species, rather than acyl-donor specificity (i.e. aliphatic vs. aromatic) (Nakayama et al. 2003). However, there are exceptions; for example, Ss5MaT2 of salvia (Salvia splendens) and Ih3AT of iris (Iris hollandica) are phylogenetically located in clusters III and V, respectively, which are distinct from the AAT-related cluster (cluster I) (Suzuki et al. 2004b, Yoshihara et al. 2006) (Supplementary Fig. S8). This implies that enzymes with similar acyl-acceptor specificities could have evolved from different ancestral proteins. A comparison of the crystal structures of BAHD members with different acyl-acceptor specificities revealed a greater diversity of main-chain fold structures around the acyl-acceptor binding sites relative to the acyl-CoA binding sites (Unno et al. 2007). This implies an evolutionary flexibility in the generation of the acceptor site structures of BAHD family enzymes.

The second type of AATs, referred to here as serine carboxypeptidase-like (SCPL)-AATs, catalyzes the transfer of acyl groups to the sugar moiety of anthocyanins using acyl-glucose as the donor molecule (Supplementary Table S3). An acylglucose-dependent-AAT (DcAASpT) activity was originally identified in carrot (Daucas carota) cell cultures in 1992 (Gläßgen and Seitz 1992) (Supplementary Fig. S9). In 2006, the cDNA encoding an aromatic AAT that transfers a p-coumaroyl group from p-coumaroyl-glucose to the glucose moiety on the 3'-position of anthocyanin (CtA3'AT) was identified (Noda et al. 2006) (Supplementary Fig. S9), and it was believed to be involved in the 3'-polyacylation of ternatin A1 in the butterfly pea. CtA3'AT is known to be a member of the SCPL protein family (termed SCPL-AT) (Milkowski and Strack, 2010). Subsequently, it was shown that, in the carnation, malylation of Pg3G takes place in a 1-O-malyl- β -D-glucose-dependent and is catalyzed by an SCPL-AT (1-Omanner malylglucose:Pg3G-6^{1/-}O-malyltransferase, DcAAMalT) (Supplementary Fig. S9) (Abe et al. 2008, Umemoto et al. 2014). The biochemical and molecular biological properties of SCPL-ATs that are involved in the biosyntheses of sinapoyl malate and sinapine have been clarified (Stehle et al. 2009, Milkowski and Strack 2010) and immunolocalization studies have shown that one of them (i.e. Arabidopsis sinapoyltransferase) was localized in a vacuole (Hause et al. 2002).



Very recently, the cDNA coding for an SCPL-AT protein (termed DgSCPL2) potentially involved in 7-polyacylation was isolated from delphinium (Nishizaki et al. 2013). DgSCPL2 was also shown to be a vacuolar protein (Nishizaki et al. 2013). Although attempts to express the enzyme in its active form in heterologous systems have not been successful, the temporal expression patterns of DgSCPL2 are similar to those of other genes encoding enzymes involved in anthocyanin biosynthesis in blue delphinium cultivars. Moreover, the defects in 7-polyacylation-related AAT activities (DgAA7GT-AT and/or DgAA7GBG-AT; see Fig. 1 and Supplementary Fig. S9) were correlated with the absence of DgSCPL2 expression in a white delphinium cultivar that accumulates an anthocyanin without 7-polyacylation (i.e. Dp3R7G) (Nishizaki et al. 2013). These results suggested that DgSCPL2 encodes an AAT that is involved in 7-polyacylation.

7-Polyacylation in Viodelphin Biosynthesis Proceeds Through Step-by-Step Elongation Using a Zwitter Donor in Vacuoles

In a previous study, 7-polyacylated viodelphin-type anthocyanin was produced enzymatically when Cy3G7G was incubated with an excess of pHBG in the crude extracts of delphinium sepals (Nishizaki et al. 2013). This is consistent with the fact that anthocyanin modification in the 7-position proceeds through the alternate glucosylation and *p*-hydroxybenzoylation catalyzed by GH1-GTs (DgAA7GT and DgAA7BG-GT) and SCPL-AT (DgSCPL2, probably identical to DgAA7G-AT and DgAA7GBG-AT) in the extracts, where pHBG serves as a common substrate for these enzymes, and acts as both the pHB and glucosyl donors, respectively (Fig. 1). pHBG most probaby occurs in vacuoles, and DgAA7GT, DgAA7BG-GT and DgSCPL2 have all been shown to be vacuolar proteins (see above). All of these observations unequivocally show that 7-polyacylation of anthocyanin takes place in vacuoles in delphinium. The bifunctionality of pHBG as a donor substrate also illustrates a novel feature in transferase enzymology, so that the donor substrate is referred to as the 'zwitter donor', as to the concept of a zwitterion (Nishizaki et al. 2013). In the delphinium petal, vacuolar concentrations of the zwitter donor greatly exceed that of the acceptor anthocyanins; pHBG and total anthocyanin contents in the petals have been measured at 3.75 and 0.15 μ mol g⁻¹, respectively (each on a fresh weight basis) (Matsuba et al. 2010). This donor/acceptor ratio should favor transfer activities of the GH1-GTs and SCPL-ATs in the vacuole. Very recently, a UGT that supplies pHBG for 7-polyacylation has been identified in the delphinium (Nishizaki et al. 2014). This enzyme, DgUpHBAGT, catalyzes the transfer of a glucosyl group from UDP-glucose to the carboxy group of pHB. It is a family 1 member of UGTs and is evolutionarily close to the cluster II UGTs (Supplementary Fig. S6).

The biosynthesis of viodelphin in delphinium has been proposed (Fig. 1) (Nishizaki et al. 2013). Dp is synthesized according to a general anthocyanin biosynthetic pathway (Fig. 2),

followed by 3-O-glucosylation and 6"-O-rhamnosylation, probably catalyzed by UAGTs, to produce Dp3R in the cytoplasm. pHBG is produced from UDP-glucose and pHBA via the action of DgUpHBAGT (probably in the cytoplasm) and transported into the vacuole. Dp3R is transported into the vacuole, where it undergoes 7-O-glucosylation catalyzed by DgAA7GT using pHBG as the glucosyl donor to produce Dp3R7G. Dp3R7G then undergoes *p*-hydroxybenzoylation that is probably catalyzed via DgSCPL2, and pHBG in the vacuole now acts as the pHB donor. The resultant product, Dp3R7BG, then undergoes a DgAA7GB-GT-catalyzed glucosyl transfer from pHBG to its terminal pHB group to produce Dp3R7GBG, followed by *p*-hydroxybenzoylation using pHBG as the pHB donor, which is probably also catalyzed via DgSCPL2.

As such, alternating glycosyl and acyl transfer reactions in the delphinium sepals are catalyzed by GH1-GTs and SCPL-ATs, respectively, using pHBG as the zwitter donor in the vacuole, producing a pHBG concatemer chain in the 7-position of Dp3R. In nature, other polyacylated anthocyanins are known to have such 'acyl-glucose concatemer' chains (e.g. ternatin A1, cinerarin and campanin; Supplementary Fig. S4). It is tempting to speculate that, during the biosynthesis of these anthocyanins, such step-by-step elongation of a concatemer chain might also take place in the vacuole using an aromatic acyl-glucose as the zwitter donor through the alternate participation of GH1-GTs and SCPL-ATs. During ternatin A1 biosynthesis in the butterfly pea, 3'-glucosylation of anthocyanin (Dp3mG) takes place in the cytoplasm via the action of CtUA3'5'GlcT (Kogawa et al. 2007; Supplementary Fig. S5). Subsequent p-coumaroylation to a 3'-glucosyl group by CtA3'AT should take place in the vacuole (Noda et al. 2006). Hence, synthesis of the remaining portions of the acyl-glucose concatemer structure in the 3'-position of ternatin A1 should also take place in a vacuole.

Perspective—Engineering of Blue Flowers Through Vacuolar Modification of Anthocyanins with an Aromatic Acyl-Glucose Concatemer Chain

Engineering of the anthocyanin pathway to accumulate the 3'or 7-polyacylated Dp-type anthocyanins in the vacuole of the petal cell is a promising approach to the engineering of truly blue flowers. A number of genes coding for enzymes (UAGTs, GH1-GTs, BAHD-AATs and SCPL-ATs) involved in the modification of the 3'- or 7-position of anthocyanins are now available, and it is possible to propose a biochemical pathway for the engineering of blue anthocyanins using the vacuolar anthocyanin modification pathways with an appropriate combination of cytoplasmic pathways (**Supplementary Fig. S10**).

In the proposed pathways, the availability of acyl-glucoses (i.e. zwitter donors) in the vacuoles of the petal cells of the host plants will be key to the success of anthocyanin engineering through this approach. In some cases, co-introduction of the *UGT* gene with *GH1-GT* and *SCPL-AT* genes might be required to supply acyl-glucose in order to achieve efficient engineering



of the anthocyanin molecules in vacuoles. The availability of transporters in the host plants that will convey the precursor anthocyanins and acyl-glucoses from the cytoplasm to vacuoles will also critically affect the successful functioning of the designed pathway. Efficient formation of metabolons (i.e. interactions among the enzymes expressed in the petal cell) might also be important factors to be considered. In the near future, using those genes might permit the generation of truly blue carnations, chrysanthemums and roses.

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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