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Review

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Comprehensive analyses of anthocyanin and related compounds to understand flower color change in ion-beam mutants of cyclamen (*Cyclamen* spp.) and carnation (*Dianthus caryophyllus*)

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Abstract We analyzed flower color mutants of cyclamen (*Cyclamen* spp.) and carnation (*Dianthus caryophyllus*) obtained by ion-beam irradiation with an idea that a comprehensive analysis of anthocyanin and its biosynthetically related compounds, such as flavonols and cinnamic acid derivatives, is necessary in order to understand flower color expression mechanism. In this review, we discuss mechanisms for flower color mutation and deduce the following ideas: anthocyanin and its biosynthetically related compounds are cooperatively and compensatively regulated; multiple factors are often concerned in the expression of the same color phenotypes; and changes in chemical structure of a pigment induces new properties that generate novel phenotypes.

Key words: Anthocyanins, carnation, co-pigments, cyclamen, ion beam.

Although ion beam irradiation has been recognized as an effective technique for plant mutation breeding, this technique tends to be thought of as 'not a scientific' procedure that depends on contingencies (Tanaka (2012) in this issue). As Tanaka described in the preface of this issue, we are trying to enhance the theoretical aspects of ion beam mutation and show that this is a 'scientific' and more efficient technique than what is imaged. The main focus of our research has targeted flower-color mutations in cyclamen (*Cyclamen* spp.) and carnation (*Dianthus caryophyllus*), whose major pigments are anthocyanins, expecting that the studies of flower color expression mechanism could lead to principle to 'theoretically' obtain mutants.

Research of the chemical structure of individual flower pigments is insufficient approach for understanding the regulation of flower color. We now understand the necessity for comprehensive analysis of anthocyanins and biosynthetically related compounds, such as flavonoids and cinnamic acid derivatives. In this review, we discuss flower color expression mechanism in ion-beam mutants, presenting the results of our comprehensive analyses. It is noted that we here use a word 'flavonoids' meaning flavones and flavonols that are so called 'colorless' flavonoids excluding anthocyanins.

Flower color is classified based on two factors: color depth and coloration. Color depth basically reflects pigment concentration. In a mutant altered in color depth, metabolic activity leading to the biosynthesis of the pigments could have been changed at any step in the pathway. Compounds partially sharing a common biosynthetic pathway with anthocyanins are flavonoids such as flavone and flavonol, and cinnamic acid derivatives such as coumaric acid and caffeic acid (Figure 1). The composition of these compounds changes corresponding to the mutated step in the biosynthetic pathway, and therefore, we can putatively assign the mutation step based on the compositional change. The

Abbreviations: Cy3G, cyanidin 3-glucoside; Cy3,5dG, cyanidin 3,5-diglucoside; Cy3MG, cyanidin 3-malylglucoside; Cy3,5cMdG, cyanidin 3,5-cyclicmalyldiglucoside; Dp3,5dG, delphinidin 3,5-diglucoside; HPLC, high performance liquid chromatography; KM, fragrant cyclamen cultivar—'Kaori-no-mai'; Mv3,5dG, malvidin 3,5-diglucoside; Pg3G, pelargonidin 3-glucoside; Pg3,5dG, pelargonidin 3,5-diglucoside; Pg3MG, pelargonidin 3-malylglucoside; Pg3,5cMdG, pelargonidin 3,5-cyclicmalyldiglucoside; TLC, thin-layer chromatography; UR, fragrant cyclamen cultivar—'Uruwashi-no-kaori'.

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second factor in flower color formation, coloration, basically is based on the chemical structure of the pigments. After addition of hydroxyl groups in the B-ring of anthocyanins, the molecule can be further modified by the addition of methyl groups, sugars and organic acids. The number of hydroxyl groups and the properties of these modifications affect the absorption spectra, resulting in changes in flower color. Therefore, we can deduce the mechanism for the color change based on the chemical structure of the anthocyanins in the mutant.

It is noteworthy that anthocyanins can reversibly change structure, thereby appearing as different colors (Goto and Kondo 1991). Generally, anthocyanin structure is stabilized to appear as a red color. As general flavonoids and cinnamic acid derivatives are colorless or pale compounds, these compounds themselves usually do not significantly affect flower color. However, in some cases, these compounds behave as co-pigments that change anthocyanin color by affecting the equilibrium state of the anthocyanin structures (Asen et al. 1972). It was reported that flower color was changed resulting from the inhibition of flavonoid biosynthesis in transgenic torenia and lisianthus plants where flavonoids served a role as co-pigments (Aida et al. 2000; Nielsen et al. 2002). These findings suggest that analysis of anthocyanin-related compounds is equally important for understanding the mechanism of flower color changes.

In this review, the relationship of flower color depth with the concentration of anthocyanins, the relationship of flower coloration with co-pigments, and the relationship of flower coloration with the chemical structure of anthocyanins is discussed.

Development of analytical technique for anthocyanins and related compounds

Flavonoids and cinnamic acid derivatives accumulate in plant tissues as sugar conjugates at relatively high concentrations of μmol/g fresh weight, a level similar to anthocyanins (Saito et al. 2006; 2007). Flavonoids and cinnamic acid derivatives are water-soluble phenolic sugar conjugates like anthocyanins (Figure 1) that may be extracted by polar solvents and are easily obtained from plant tissues together with anthocyanins.

Whereas anthocyanins have characteristic absorption spectra in the visible light spectrum, flavonoids and cinnamic acid derivatives have characteristic absorption spectra in the ultraviolet. Information about the chemical structures of these compounds may be obtained based on their spectral properties. High performance liquid chromatography (HPLC) coupled to photodiode-array detector permits the monitoring of absorption spectra at multiple wavelengths. Thin-layer chromatography (TLC) is another available technique for the analysis of phenolic compounds. Under ultraviolet light, flavonoids absorb the light and show a dark image, whereas cinnamic acid derivatives irradiate bluish white fluorescent light; these compounds are easily detected under ultraviolet light after TLC (Figure 1). Furthermore, flavonoids can be non-destructively detected in living tissue under ultraviolet light, and sometimes the distribution pattern of these compounds can also be observed (Fukuta et al. 2005; Nakayama et al. 2006).

To study molecular interactions, each compound must be separated and purified by chromatography, and then these purified compounds must be mixed in a test tube to monitor their interactions. Compounds whose structural and chemical properties are known are better objects for this type of analysis. Therefore, well-known compounds tend to become targets for interaction studies, including anthocyanin-co-pigment interactions. Such research behavior prevents the discovery of more novel types of interactions.

When co-pigments separate from anthocyanins, anthocyanin color changes from purple to red on TLC. This result indicates that the molecular interaction between anthocyanin and the co-pigment occurs on the TLC matrix as well as in the test tube, and the occurrence of co-pigments can be indicated by color change of the anthocyanin image on TLC. Utilizing the property, we detected a structurally and functionally novel co-pigment; the deduced structure is out of the conventional categories of co-pigments and causes a paler color change that is contrary to the effects of conventional co-pigments (Shimizu-Yumoto et al. 2012). We now believe that co-pigment research should be carried out without prediction of structure and function. As described later, we also found that co-pigments are involved in the color of cyclamen flowers based on coloration change of anthocyanin image on TLC.

A lighter color mutation occurs from a decrease in anthocyanin content with an increase in other compounds

Because mutations induced by ion-beam irradiation generally delete functions operating in an organism, this mutagen is effective in changing the concentrations of target metabolites (Tanaka 1999; Tanaka et al. 2010). Inhibition of enzyme activity involved in anthocyanin biosynthesis causes a decrease directly in anthocyanin concentration. In the white marginal tissue of a *Petunia* picotee flower, expression of chalcone synthase, an anthocyanin biosynthetic enzyme, was specifically repressed by endogenously operating post-transcriptional gene silencing (Saito et al. 2006; Morita et al. 2012). In addition to decreasing the anthocyanin concentration, a significant increase in the concentrations of caffeoyl glucosides and coumaroyl glucoside were found in white flower tissue compared with colored flower tissue (Saito et al. 2006; Saito et al. 2007). Anthocyanins and these cinnamic acid

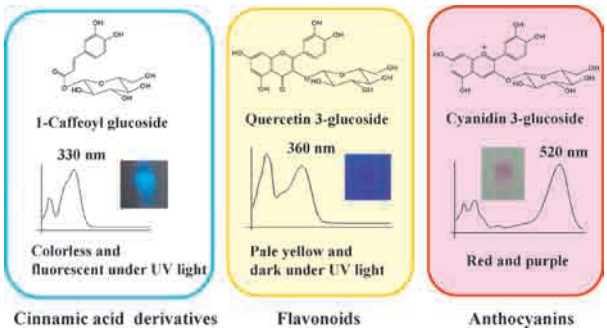

Figure 1. Properties of anthocyanins, flavonoids and cinnamic acid derivatives. Their representative chemical structures, absorption spectra, λ_{max}, and images under visible light (anthocyanin) or ultraviolet light (flavonoid and cinnamic acid) are presented.

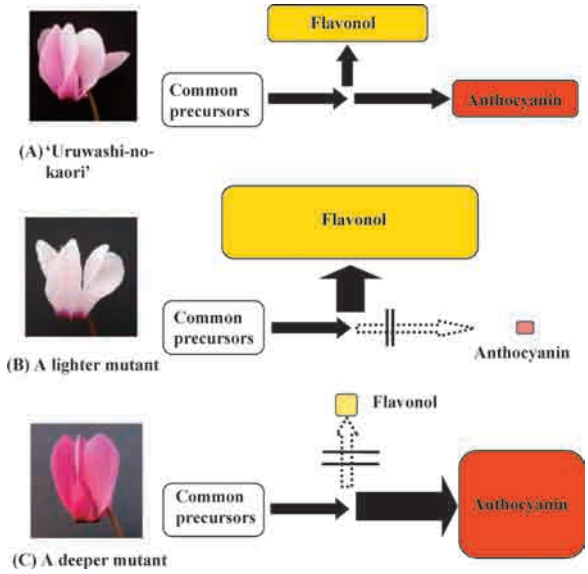

Figure 2. Generation mechanism of lighter and deeper color mutants of cyclamen. (A) The original plant 'Uruwashi-no-kaori' (UR). (B) A a lighter mutant. (C) A deeper mutant. Biosynthesis of anthocyanin is inhibited, and instead, metabolism of the common precursor into flavonol is activated (B). Biosynthesis of flavonol is inhibited, and instead, metabolism of the common precursor into anthocyanin is activated (C). Photographs of A, B and C are the same ones presented in Figure 2 of a review described by Ishizaka et al. (2012) in this issue.

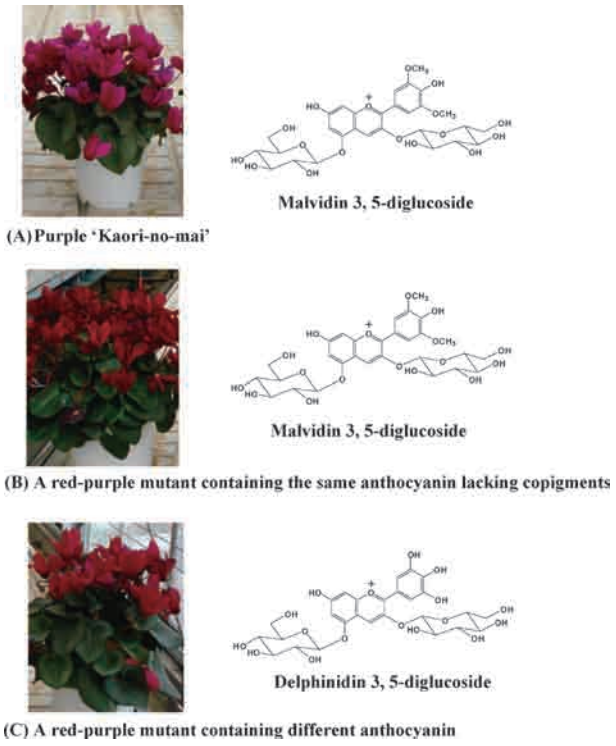

Figure 3. Red-purple coloration mutants derived from the purple-flowered cyclamen 'Kaori-no-mai' (KM) by ion-beam irradiation. (A) KM. (B and C) Two flower coloration mutants with the same color of flowers. One mutant (B) had the same anthocyanin, malvidin 3, 5-diglucoside (Mv3,5dG), as the original plant (A) but lacked flavonols. The other mutant (C) had a different anthocyanin, delphinidin 3,5-diglucoside (Dp3,5dG), but had the same flavonols as the original plant (A). Some of the flavonols functioned as co-pigments with Mv3,5dG. Lack of co-pigment and change in anthocyanin structure caused the mutations of (B) and (C), respectively.

derivatives are synthesized in common pathway before synthesis of chalcone. Inhibition of the biosynthesis of chalcone decreased concentration of anthocyanin and increased concentration of cinnamic acid derivatives, compensatively. Based on these compositional changes, the mutated step in the metabolic pathway could be assigned.

'Uruwashi-no-kaori' (UR) is a fragrant cyclamen cultivar with pink flowers that was generated by interspecific hybridization of two horticultural species *Cyclamen persicum* and *C. purpurascens* (Figure 2A) (Ishizaka and Uematsu 1995a; 1995b; Ishizaka et al. 2002; Ishizaka 2008). Mutants with pale pink flowers were obtained by ion-beam irradiation (Figure 2B). In addition to a decrease in anthocyanin concentration, an increase in flavonol concentration was found in the flower of this mutant compared with non-irradiated UR. Dihydroflavonol is a common precursor of flavonol and anthocyanin. These compositional changes suggest that the activity of dihydroflavonol 4-reductase and/or anthocyanidin synthase, which catalyzes the conversion of dihydroflavonol into anthocyanin, is reduced in the mutant, resulting in a decrease in anthocyanins and an increase in flavonols.

A deeper color mutation occurs from an increase in anthocyanins and a decrease of other compounds

In marginal colored tissue of petunia picotee flowers, expression of flavonol synthase, a biosynthetic enzyme of flavonol from dihydroflavonol, was specifically repressed (Saito et al. 2006). In addition to an increase in anthocyanin concentration, a decrease in the concentration of flavonols was found in the colored flower tissue compared with the white flower tissue (Saito et al. 2006; 2007). Similar phenomenon was

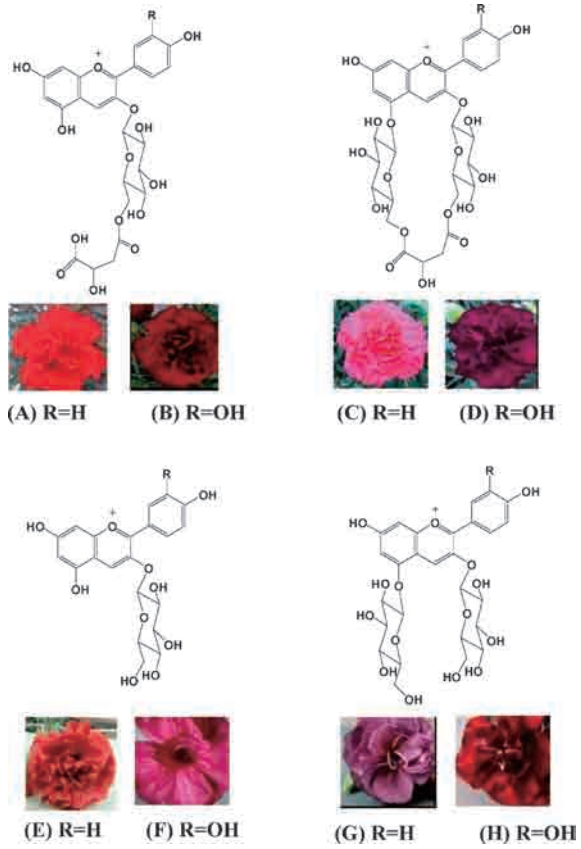

Figure 4. Structures of anthocyanins contained in carnation flowers and flower colors corresponding to each anthocyanin. (A) Pelargonidin 3-malylglucoside (Pg3MG). (B) Cyanidin 3-malylglucoside (Cy3MG). (C) Pelargonidin 3,5-cyclicmalyldiglucoside (Pg3,5cMdG). (D) Cyanidin 3,5-cyclicmalyldiglucoside (Cy3,5cMdG). (E) Pelargonidin 3-glucoside (Pg3G). (F) Cyanidin 3-glucoside (Cy3G). (G) Pelargonidin 3,5-diglucoside (Pg3,5dG). (H) Cyanidin 3,5-diglucoside (Cy3,5dG). Acylated anthocyanins (A–D) express general cultivar colorations while non-acylated anthocyanins (E–H) corresponding to A–D express peculiar brilliant-metallic-duskish colorations.

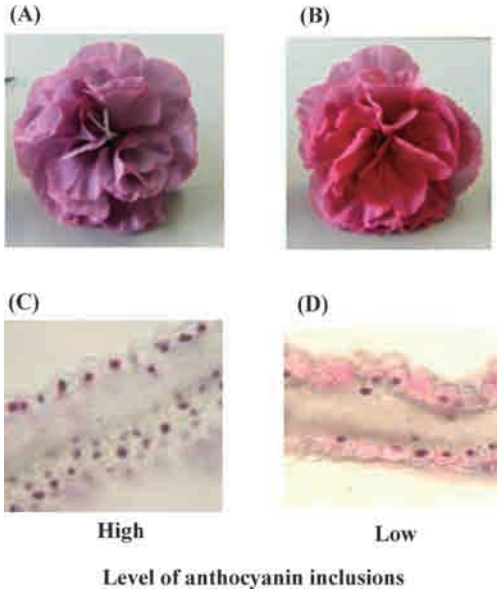

Figure 5. Peculiar coloration mutants of carnation. (A) A blue-purple mutant showing glittering coloration. (B) A red-purple mutant derived from (A). (C) Epidermal cell of (A) containing anthocyanin in the vacuoles. (D) Epidermal cell of (B) containing anthocyanin in the vacuoles. Each mutant has the same non-acylated anthocyanin, pelargonidin 3, 5-diglucoside (Pg3,5dG). The difference is in the level of anthocyanin inclusions in vacuoles, where (B) has less anthocyanin inclusion than (A).

reported by Holton et al. (1993) and Davies et al. (2003); inhibition of flavonol synthase by introduction of the antisense transgene construct caused deeper color change of petunia flowers. A decrease in the metabolism of dihydroflavonol into flavonols probably causes activation of metabolism into anthocyanin, resulting in the increased anthocyanin concentration and formation of colored tissue. This interpretation is based on a concept of cooperative and compensative regulation among anthocyanins and related compounds that when one compound decreases in abundance from the loss of biosynthetic activity, other compounds sharing biosynthetic pathway increase.

A deep pink flower mutant was also obtained from UR by ion-beam irradiation (Figure 2C). In addition to an increase in anthocyanin concentration, a decrease in the concentrations of flavonols was found in flowers of the deep pink mutant compared with UR. Similar to marginal colored tissue of the *Petunia* picotee flower, a decrease in the metabolism of dihydroflavonol to flavonol probably caused an increase in the mutant's anthocyanin concentration. Our research has shown that mutation by ion-beam irradiation generates both lighter and deeper color mutants that are capable of elucidating the mutation mechanism.

Color changes by a decrease in co-pigments

'Kaori-no-mai' (KM) is another fragrant cyclamen cultivar generated by the same interspecific hybridization as UR and has purple flowers (Figure 3A). Some mutants with red-purple flowers were obtained from KM by ion-beam irradiation (Figure 3B). Changes in anthocyanin structure are one of the general causes of flower color change; however, one of the red-purple mutants and KM had the same anthocyanin composition of malvidin 3,5-diglucoside (Mv3,5dG) as the major anthocyanin that was present at a similar concentration. These data were obtained by HPLC analysis.

We analyzed the occurrence of co-pigments on purple flowers of KM by TLC as described in the previous section. The results indicated that some flavonols contribute to the purple coloration as co-pigments for Mv3,5dG. HPLC analysis clearly indicated that the concentration of flavonols including these co-pigments significantly decreased in the red-purple mutant. Neither changes in chemical structure nor the concentration of anthocyanin pigment caused the change in flower color,

but the inhibition of flavonoid co-pigment biosynthesis probably caused the flowers to change from purple to red-purple in this mutant. Similar reddish flower coloration change was reported in lisianthus, where flavonol synthase was inhibited by introduction of the antisense transgene construct (Nielsen et al. 2002).

The major flavonoids of KM are composed of more than five kinds of compounds. Each of these compounds has been annotated to be either kaempferol glycosides or quercetin glycosides. These co-pigment flavonols had relatively high R_f values on cellulose TLC developed with 10% acetic acid. We try to identify co-pigment flavonols among previously reported flavonols in cyclamen plants (Miyajima et al. 1990; Van Bragt 1962; Webby and Boase 1999).

A color change due to a lack of methylation in anthocyanin

The functional groups significantly involved in coloration are the hydroxyl groups and their methylated forms in the B-ring of the anthocyanin structure. Another red-purple flower color mutant was obtained from ion-beam irradiated mutants of KM (Figure 3C) (Kondo et al. 2009). The major anthocyanin of this mutant was found to be delphinidin 3, 5-diglucoside (Dp3,5dG). This mutant lacked the enzyme catalyzing methylation of the hydroxyl groups in the B-ring of anthocyanin so that biosynthesis of Mv3,5dG was inhibited and the substrate, Dp3,5dG, accumulated (Akita et al. 2011). The major anthocyanins of *Cyclamen* spp. were believed to be restricted to malvidin, peonidin and cyanidin types (Sugiyama et al. 1997; Webby and Boase 1999). Our report was the first to breed a *Cyclamen* containing delphinidin-type anthocyanins as the major anthocyanin of the flower. This finding has both horticultural and biological importance for cyclamen plants.

Dp3,5dG has a little longer λ_{max} than Mv3,5dG. The color of Dp3,5dG may be bluer than that of Mv3,5dG. No significant difference was found in the flavonol composition in this mutant. Therefore, we assumed that flavonols operating as co-pigments for Mv3,5dG could not function as a co-pigment for Dp3,5dG. This mutation is probably caused by a change of anthocyanin structure and the lack of a flavonol co-pigment effect for the new anthocyanin. Combinational conditions between anthocyanins and co-pigments are not well characterized. Our finding indicates that this is an important matter to be solved in order to understand the mechanisms for flower color development.

Peculiar flower color change by anthocyanin inclusion

Carnation flowers contain the following four kinds of major anthocyanins, all of which have glucose moieties acylated by malic acid: pelargonidin 3-malylglucoside (Pg3MG) (Figure 4A), cyanidin 3-malylglucoside (Cy3MG) (Figure 4B), pelargonidin 3,5-cyclicmalyldiglucoside (Pg3,5cMdG) (Figure 4C), and cyanidin 3,5-cyclicmalyldiglucoside (Cy3,5cMdG) (Figure 4D) (Bloor 1998; Nakayama et al. 2000; Terahara et al. 1986; Terahara and Yamaguchi 1986). Each anthocyanin is responsible for a specific flower color (Figure 4) as described in another review described by Okamura et al. (2012) in this issue. In our work, mutants containing a non-acylated anthocyanin corresponding to each of the carnation flower anthocyanins, pelargonidin 3-glucoside (Pg3G) (Figure 4E), cyanidin 3-glucoside (Cy3G) (Figure 4F), pelargonidin 3,5-diglucoside (Pg3,5dG) (Figure 4G), and cyanidin 3,5-diglucoside (Cy3,5dG) (Figure 4H), were obtained by loss of the acylation enzyme (Abe et al. 2008) and glucosyltranferase (Matsuba et al. 2010) activities by ion-beam irradiation (Okamura et al. 2003). The flowers of each mutant show a peculiar glittering coloration of purple (Figure 4E), bronze red (Figure 4F), blue-purple (Figure 4G) and dark red (Figure 4H), respectively. All of these mutants have an inclusion of anthocyanin in the vacuoles of petal epidermal cells. Occurrence of the inclusion seems to be the cause of the peculiar color (Gonnet and Hieu 1992; Markham et al. 2000).

Whereas acylation by aromatic acids like caffeic acid and coumaric acid can change anthocyanin color operating as an intra-molecular co-pigment, acylation by aliphatic acids like malonic acid and acetic acid rarely influence coloration. However, at least in carnation flower, lack of acylation by malic acid gives inclusion properties to anthocyanin and results in peculiar coloration (Markham et al. 2000). These non-acylated anthocyanins are widely found in many plants. For example, Pg3,5dG and Cy3,5dG are the major anthocyanins of rose flowers. Generally these anthocyanins are dissolved and an inclusion is not formed in the vacuoles of these flowers. These results suggest that carnation has a potentially unique mechanism for forming inclusions of non-acylated anthocyanin.

We obtained another color mutant (Figure 5B) from the blue-purple Pg3,5dG type mutant (Figure 5A) by ion-beam irradiation. The new mutant had red-purple flowers and kept its glittering coloration. The mutant also had Pg3,5dG as a unique major anthocyanin but had less inclusion than the original mutants (Figure 5C, D). Ion-beam irradiation can eliminate other factors causing the formation of anthocyanin inclusions and increasing the variation of the peculiar coloration of carnation flowers.

Conclusions

Here, we summarize the ideas obtained from our successive ion-beam mutant studies that can be generally applied to the generation of mutants as follows:

(1) Because of cooperative and compensative biosynthetic regulation between a target and its related compounds, mutants in which the target compounds either increased or decreased could be generated by ion-beam irradiation.

(2) When multiple compounds are concerned in the expression of one phenotype, different types of mutants occur among the same phenotype.

(3) Structural changes of the target compound influence the physical, chemical and physicochemical properties, such as light-absorption, co-pigmentation effect and solubility, respectively, resulting in the acquisition of a novel phenotype.

We conclude that as Tanaka (2012) discussed in the preface of this issue, now we can predict the color of ion-beam mutant to some degree based on the above ideas and information of anthocyanins and related compounds in the original plants.

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