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Review

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Breeding glittering carnations by an efficient mutagenesis system

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Abstract We have developed a systematic and directed method to create novel glittering mutants in carnation (*Dianthus caryophyllus*) by combining the advantages of ion-beam breeding and genomic information. The method is a series of steps that include: (i) establishing a basic strategy to select appropriate genotypes for specific breeding aims using genomic information, (ii) identifying factors that induce anthocyanic vacuolar inclusions (AVIs), (iii) using ion-beam irradiation consecutively to modify pigment glycosylation and/or acylation, (iv) tuning shading of color, and (v) selecting stable mutants with markers. During the course of this work, we identified a factor that causes AVIs and analyzed the content of anthocyanins and related compounds in the flowers of these mutants. Applying the method, we have created two highly novel carnations with the most glittering color ever reported.

Key words: Anthocyanin, carnation, ion-beam irradiaton, mutation breeding, glittering.

Ion-beam breeding, a newly established breeding technique, has become an important tool for producing novel and unique mutants that cannot be obtained by conventional mutagenesis methods (Okamura et al. 2003; Tanaka et al. 2010). Flower color is an important characteristic in the floriculture industry, and novel colors create a positive economic impact. A wide variety of flower colors can be derived from only six types of anthocyanin aglycones that change color after modification with glycosyl and/or acyl moieties. For breeding colors in otherwise superior cultivars, mutation breeding especially with ion beams is among the best choices because it alters only a few traits and retains other characteristics of the original cultivars. In this review, we describe measures to select appropriate materials to make desired mutants using genomic information, improved procedures of ion-beam irradiation, and their application to carnation (*Dianthus caryophyllus*), one of the world's most important floricultural crops. We also report our success in creating highly novel floricultural varieties; that is, carnations with glittering colors in the true sense of the word. In addition, several factors relevant to the novel phenotypes have been discovered such as pigment constituents, genes and morphological characteristics of petal cells.

Basis for breeding glittering carnations using genomic information and mutagenesis

Carnation varieties have been created mainly by cross-breeding, partially combined with spontaneous mutation. However, due to its heterozygous genetic background, being heterozygous at many different loci throughout the genome, conventional cross breeding often produces a huge number of undesired individuals in segregating population. Therefore, it is quite laborious to obtain a desired individual that combines the good characteristics of the parents used in the cross. Moreover, it is quite rare to improve only a few traits in otherwise superior varieties. Now, genetic manipulations have been successfully applied to develop a blue carnation containing delphinidin, a pigment that typical carnations do not have (Tanaka et al. 2008). Although genetic engineering by recombinant DNA techniques is a promising way to produce attractive varieties, the technology is expensive and takes much time to create varieties with the desired characteristics and to evaluate their influence on the ecosystem according to the Cartagena Protocol on Biosafety. Mutation breeding with ion-beam irradiation is an alternative approach for addressing the problems described above because ion beams deposit high energy on a local target, thereby inducing a limited number of large and irreparable

Abbreviations: AMalT, anthocyanin malyltransferase; AVIs, anthocyanic vacuolar inclusions; Cy3G, cyanidin 3-glucoside; Cy3,5dG, cyanidin 3,5-diglucoside; Cy3MG, cyanidin 3-malylglucoside; Cy3,5cMdG, cyanidin 3,5-cyclicmalyldiglucoside; GST, glutathione *S*-transferase; Pg3G, pelargonidin 3-glucoside; Pg3,5dG, pelargonidin 3,5-diglucoside; Pg3MG, pelargonidin 3-malylglucoside; Pg3,5cMdG, pelargonidin 3,5-cyclicmalyldiglucoside.

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DNA deletions and change only a few characteristics of otherwise well-adapted plant cultivars.

The major cyanic pigments of carnation are anthocyanins, consisting of pelargonidin 3-malylglucoside (Pg3MG), pelargonidin 3,5-cyclicmalyldiglucoside (Pg3,5cMdG), cyanidin 3-malylglucoside (Cy3MG), and cyanidin 3,5-cyclicmalyldiglucoside (Cy3,5cMdG). In carnation, the relationship of anthocyanin pigments to floral colors is very simple; the color of a cultivar is due to one main anthocyanin. In fact, cultivars having Pg3MG, Pg3,5cMdG, Cy3MG, and Cy3,5cMdG as their main anthocyanin pigment have flowers that are red, pink, dark red and purple, respectively. Along with the difference of anthocyanin that accumulates in the petal vacuoles, factors such as co-pigmentation, vacuolar pH and metal ions make diversity of colors. The glutathione *S*-transferase (GST) activity is necessary for vacuolar uptake of anthocyanins (Mehlquist and Geissman 1947), which we suppose affects the shading of flower colors in carnation. In addition, shape of petal epidermal cells affects the intensity and shading of colors (Noda et al. 1994).

Another unique hue in some flowers is dusky colors. Whereas most carnations with pink or red flowers show plain colors, a cultivar named 'Nazareno' has flowers with dusky purple. Microscopic analysis of the petals revealed that the dusky color is caused by the formation of anthocyanic vacuolar inclusions (AVIs). We studied the structure of the anthocyanin in AVIs and found it to be pelargonidin 3,5-diglucoside (Pg3,5dG) without a malyl moiety (Ozeki et al. 2011). Then, we extensively examined the relationship between AVIs and genotypic background using cultivar breeding and genetic analysis, and found one factor causing the dusky purple color in 'Nazareno' (Umemoto et al. 2009). An enzyme, anthocyanin malyltransferase (AMalT), turned out to be the factor (Abe et al. 2008), without which petals look dusky purple (Table 1). However all carnation flowers with AVIs reported thus far are not glittering but dusky with some metallic hue, so they do not glitter in the true sense. We tried to create glittering carnation by altering some characteristics of the potential materials.

Ion beams can alter a few characteristics of otherwise well-adapted plant cultivars and become an effective tool for generating mutations in crops. A great impact of ion beam breeding on plant seed and seedling business has been demonstrated by such an example as breeding a series of carnations in the joint R&D program between Kirin Holdings Co., Ltd. and the Japan Atomic Energy Agency (JAEA). The carnation varieties have been commercialized in Japan and Europe, resulting in a flower wholesale market value of more than 450 million yen per year (Okamura et al. 2006). We have also developed a directed mutagenesis method to enhance the frequency and variation of flower color mutants by a combination of ion-beam irradiation and pre-treating materials that lead to changes in the gene expression profile (Hase et al. 2010). By exploiting the sophisticated method described above, carnations with glitter colors are expected to be created.

Efficient genotype selection using genomic information and the creation of glittering carnations—Breeding of glittering carnation from a line '07MC4' by ion-beamed mutagenesis of AMalT allele

There are two important prerequisites to succeed in breeding excellent varieties with glittering colors. The first is to obtain materials with superior characteristics such as disease resistance, high productivity, etc. The second is to identify genotypes that have potential to change into glittering genotypes from among the superior materials.

In total, 1,550 genotypes of carnation cultivars and breeding lines were analyzed by means of crossing and genetic information, and eighteen genotypes were selected that presumably lack AMalT activity in one allele of their diploid genome. Subsequently, their disease

Table 1. Detection of anthocyanin malyltransferase (AMalT) activities in twenty carnation genotypes of various flower colors. AMalT, active; amalt, no activity.

Cultivars, Breed (Genotypes)	Flower color	AMalT amalt	Cultivars, Breed (Genotypes)	Flower color	AMalT amalt
Rose candle	rose	AMalT	Boilot	yellow speckled	AMalT
Crowdie	rose	AMalT	**Nazareno***	dusky metallic	amalt
Cosmo Cherry	rose fancy	AMalT	Custard R	rose	AMalT
Naja	rose fancy	AMalT	WS01-848	rose	AMalT
Live	purple	AMalT	Mo 001M	rose fancy	AMalT
Garnet	deep red	AMalT	**2005MA3***	dusky metallic	amalt
Domas	rose	AMalT	2004FC4	rose fancy	AMalT
Cinderella	rose	AMalT	H205043	rose	AMalT
Celta*	dusky metallic	amalt	H205064	rose	AMalT
Rosalba	rose	AMalT	PV7746C	rose	AMalT
Dover	white	AMalT	2006MC7	rose	AMalT

* All three genotypes with AVIs lack AMalT activity.

resistance and productivity were examined, and one breeding line '07MC4' was finally selected. The line has cherry petals and comes from cross between LEK04 and Nazareno; the female parent 'Nazareno' has dusky purple color with AVIs, indicating that one allele of AMalT is lacking in '07MC4'. If we destroy the AMalT gene in the other allele by mutation, the resulting mutants are expected to show AVIs and have dusky or glittering flowers.

For this purpose, ion-beam breeding is an excellent tool because it has the ability to induce point deletions by relatively far smaller amounts of irradiation than other types of ionizing radiations.

Small leaf segments with micro buds were irradiated by ion beams in the irradiation chamber connected to a vertical beam line from the Azimuthally Varying Field cyclotron at JAEA (Takasaki, Gumma, Japan). Other procedures are the same as those described by Okamura et al. (2003, 2006). Among 1,800 plants derived from 15–20 Gy irradiations of 320 MeV carbon ion beams, one plant was selected that had glittering reddish purple flowers and was named '07MGRP'. This is the first demonstration of intentional generation of glittering mutant. The selected mutant has lighter color than 'Nazareno' and glitters (Figure 1A).

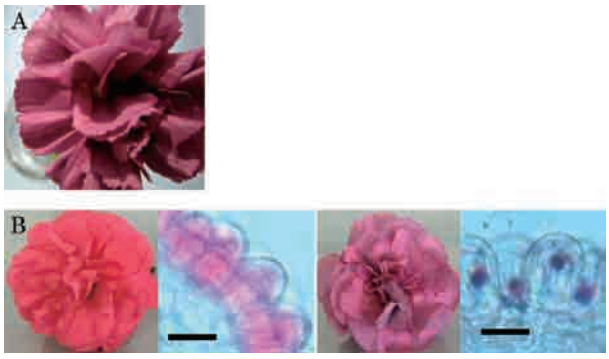

Figure 1. (A) Flower of '07MGRP' with glittering reddish purple petals produced with ion beam irradiation of cherry '07MC4'. (B) Flowers and petal cells of 'La Vie en Rose' or 'LAV' (left) and its mutant 'LAVA' (right). The anthocyanin of 'LAV' is soluble in vacuoles, and that of 'LAVA' forms AVIs in vacuoles. Bars, 30 μm.

Figure 2. Flowers and petal cells in 'Nazareno' (left) and its silver mutant 'Nazareno MA' (right). Both 'Nazareno' and 'Nazareno MA' have pelargonidin 3,5-diglucoside as their sole colored pigment. The average sizes of AVIs in 'Nazareno' and 'Nazareno MA' are 15 μm and 7 μm in diameter, respectively. Bars, 30 μm.

Breeding of dusky glittering carnation 'LAVA' from the line 'LAVS'

Twelve carnation cultivars with increasing sales were first selected, and then their flowers in numerous greenhouses were extensively examined by visual observation to determine if the flowers could change into flowers with AVIs. One cultivar named 'La Vie en Rose' or 'LAV', was discovered that shows dusky grey sectors in some flowers. The cultivar 'LAV' has superior characteristics in general. We further examined more than 10,000 'LAV' individuals in greenhouses and selected one individual that has a number of AVIs sectors. We named the individual 'LAVS'.

In vitro cultures of the carnation line 'LAVS' were established. *In vitro* nodes were irradiated by 10 Gy 320 MeV carbon ion beams. Irradiated buds were grown to 5 cm, and then cutback three times to produce 500 individual plants in total. We obtained twenty-two individual lines that had solid glittering petals with AVIs. Thirty individuals of each line, i.e. 660 individuals in 22 lines were examined for their characteristics during flowering. Finally, we selected the best line, named 'LAVA', that has solid glittering flowers with AVIs and the excellent characteristics of 'LAV' as well (Figure 1B).

Making 'Nazareno' to glitter by tuning shading with ion-beam irradiation

Although 'Nazareno' has stable AVIs in all the petals, its flower color is not glittering purple but dusky purple. We hypothesized that the petal cells of 'Nazareno' had a surplus amount of pigments responsible for the dark color. Condensation of pigments is likely to be another important factor for creating the glittering effect, for we suppose that a transparent area in the vacuoles serves like a diamond to reflect light from deep within the cells, resulting in glittering flowers. When we tune the size of AVIs, flowers with glittering colors in the true sense of the word might be obtained.

To confirm this idea, we tried ion-beam breeding of 'Nazareno' and have succeeded in developing a new line with purplish silver flowers. We named the line 'Nazareno MA' and investigated a mechanism that changes dusky 'Nazareno' into silver 'Nazareno MA'. Petals of typical flowers from both genotypes were sliced into 110 μm sections and examined with a microscope. Whereas cells of 'Nazareno' and 'Nazareno MA' have Pg3,5dG as their sole colored pigment, the size of AVIs differs significantly between the two. The average size of AVIs in 'Nazareno' and 'Nazareno MA' are 15$\pm$2 μm and 7$\pm$1 μm in diameter, respectively. The silver color of 'Nazareno MA' was caused by the downsizing of AVIs in 'Nazareno' (Figure 2).

Downsizing of AVIs in 'Nazareno MA' is supposed to be caused by decrease in the amount of anthocyanin, so we investigated the gene for GST that is responsible for anthocyanin transport to vacuoles in both genotypes

Table 2. Flower color mutants obtained by consecutive irradiation of 320 MeV carbon ion beams to 'LAVA'*

Dose	Color mutants (Number) Glittering	Number tested	Mutation rates (%)
20 Gy	white (2), light pink (2), glittering red (5), glittering salmon (2),	460 individuals	2.4
15 Gy	dark salmon (1), glittering purple (3), glittering deep purple (5)	460 individuals	2.0
10 Gy	light pink (2), glittering purple (2), glittering deep purple (1)	400 individuals	1.3

*'LAVA' was produced by ion-beam irradiation of cultivar 'LAV'.

Figure 3. Flowers (upper) and petal cells (lower) of 'LAVA Purple', 'LAVA Red', 'LAVA Salmon' and the original cultivar 'LAV' (from left to right). 'LAVA Purple', 'LAVA Red', and 'LAVA Salmon' are ion-beamed mutants of 'LAVA' which in turn is an ion-beamed mutant of 'LAV'.

following the methods of Sasaki et al. (2012, in this issue). The study revealed that ion beams did not destroy the GST gene in 'Nazareno MA'. The same level of GST gene expression was detected in both 'Nazareno' and 'Nazareno MA' by reverse transcription-PCR, and there are no nucleotide substitutions in GST cDNAs in the petals of either genotype (Sasaki and Nishizaki, personal communication). The decrease in AVIs size is due to mechanisms other than GST transcription such as loss of other pigmentation genes that cause less production and/or accumulation of anthocyanin.

We believe tuning shading of colors into lighter one is one important technique to create glittering carnations because silver color of 'Nazareno MA' is, we suppose, due to its far lighter color than dusky 'Nazareno'. The example described above shows that tuning shading of petal colors by ion-beam breeding is possible and predictable.

Making a wide range of glittering colors in carnation—Consecutive use of ion beam irradiation to make a range of glittering colors in carnation

Ion beams deposit high energy locally and densely on the genomic DNA resulting in a few changed traits without other undesired alterations. This feature is ideal for consecutive use for altering one or a few characteristics in superior cultivars. We confirmed the merits by making a wide range of glittering colors in carnation line 'LAVA' that had been produced by ion beam irradiation.

In vitro nodes of 'LAVA' were irradiated with the 320 MeV carbon ion beams. Preliminary irradiation

Figure 4. Flowers and petal cells of breeding line 'FA03' (left) and its ion-beamed mutant 'FA31' (right). The round shape of the epidermal cells in the petal of the original genotype, 'FA03', turned into conical-shaped cells in the mutant, 'FA31'. Bars, 30 μm.

Figure 5. Flowers and petals of breeding lines, 'MA21 Red (left)' and 'MA21 Purple (right)'. Their glittering colors are unique and highly novel; their flowers glitter more than any other carnations reported thus far.

experiment of the variety showed that shoot growth decreased with increasing dose and the median growth dose was estimated to be 20 Gy. Subsequently we used irradiation doses of 10–20 Gy for experiments. Plants were obtained from axillary buds of the nodes, and cutback three times as described in Section 3-2 to produce 460, 460, and 400 individuals from cultures irradiated with 10, 15 or 20 Gy, respectively. Number of flower color mutants derived from the irradiation is shown in Table 2. The mutation frequencies in irradiations of 20 Gy, 15 Gy and 10 Gy were 2.4%, 2.0% and 1.3%, respectively. Flower color mutants such as white, light pink and dark salmon flowers with a bit glittering traits were obtained, whereas eleven solid glittering mutants of new colors have been created; five glittering red, two glittering salmon, five glittering purple, and one glittering deep purple individuals (Table 2).

Typical individuals of the colors glittering purple, glittering red and glittering salmon have been selected as 'LAVA Purple', 'LAVA Red', and 'LAVA Salmon', respectively. We microscopically examined the petal cells of these mutants and found that the degree of pigment inclusion, or condensation, differs between the mutants. Petals of both 'LAVA Purple' and 'LAVA Red' have AVIs in almost all the epidermal cells but 'LAVA Salmon' in about half of them, and AVIs of 'LAVA Salmon' are

smaller than other glittering lines (Figure 3). Among these twenty-five mutants, only five, two white and three light pink, showed growth reduction when compared with 'LAVA'. The others, that is 80% of the mutants, have good vigor, strong stems and disease resistance that are comparable to both 'LAVA' and 'LAV'.

This is the first demonstration of intentional breeding of a range of glittering colors in carnation. The results clearly indicate that consecutive irradiation with ion beams is effective and efficient for improving a few characteristics in otherwise excellent cultivars.

Analysis of anthocyanins and related compounds in the glittering mutants

Comprehensive analysis of pigments and related compounds will make it possible to deduce genes that are responsible for a range of glittering colors.

We analyzed anthocyanins and related compounds in three glittering mutants, 'LAVA Purple', 'LAVA Red' and 'LAVA Salmon', and their original cultivar 'LAV' (Figure 3), by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). Analysis of anthocyanins and related compounds revealed that 'LAV' has Pg3,5cMdG, whereas 'LAVA Purple' and 'LAVA Red' have pelargonidin 3,5-diglucoside, Pg3,5dG, and pelargonidin 3-glucoside, Pg3G, respectively. This result shows that 'LAVA Purple' is lacking acylation enzyme activity (Abe et al. 2008), and 'LAVA Red' is lacking both acylation and glucosyltransferase enzyme activities (Matsuba et al. 2010). The novel mutant 'LAVA Salmon' with glittering salmon petals contains a unique phenolic compound; that is, chalcone in addition to Pg3G. The reddish glittering color of Pg3G in AVIs combined with the yellow color of chalcone in vacuoles generated unique glittering salmon color in this ion-beamed mutant (Figure 3).

Individuals with glittering red and glittering salmon were obtained from the irradiation of 20 Gy only, indicating that relatively high dose might be preferable to change the structure of anthocyanins.

Altering the shape of petal cells—Another way to increase glittering

Flower color is mainly determined by the production of anthocyanins, but the shading and intensity of the color are affected by other factors such as vacuole components, pH and ions. The shape of petal epidermal cells is reported to affect color intensity of flowers (Noda et al. 1994). This should be true in carnation. We examined the possibility of ion beam irradiation to make glittering carnations. The line 'FA03', fancy petals with dusky pinkish purple (Figure 4), is used as a material for the irradiation. 'FA03' is a superior individual selected from segregants between 'FCh03', fancy cherry breeding line, and 'Nazareno'. Procedures for mutagenesis are the same as those described by Okamura et al. (2003, 2006). Among 2,120 individuals derived from ion-beamed plants, we observed dozens of mutants with glittering colors. Then one individual with a novel color, glittering fancy purple, was selected and named 'FA31'.

We examined petals of 'FA31' by microscope, and found that all the cells have a conical shape. In addition, 'FA31' has highly condensed small AVIs in its petals (Figure 4). As described in Section 4, downsizing of AVIs affects shading of petal color, resulting in purplish silver glitter in 'Nazareno MA'. Downsizing of AVIs in 'FA31' certainly cause it to glitter to some degree. 'Nazareno MA' has light color that is relatively easy to glitter. Petals of both dusky 'Nazareno' and glittering 'FA31' have dark hues, which are unlikely to produce glittering colors. Still 'FA31' glitters in the true sense, indicating that conical shape of the line has substantial effect on its glittering characteristics.

Considering that 'FA31' was produced by ion-beam breeding, the mutant is likely to be caused by a mutation of the counterpart to the *Antirrhinum* gene reported previously (Noda et al. 1994). Although the genes responsible for the shape change in the petal cells of 'FA31' remain to be studied further, it is obvious that irradiation with ion beams can cause alterations of not only the contents of pigments but also the shape of petal cells.

Creation of highly novel glittering flowers: systematic use of the efficient mutagenesis

As described above, we have demonstrated that mutagenesis with ion beams can alter several factors affecting flower color: the composition and amount of anthocyanins and related compounds, the size of AVIs and the morphology of petal epidermal cells. We have also demonstrated that consecutive use of ion beam irradiation is effective for step-by-step improvements in creating novel flowers with excellent characteristics. We used this formula to create highly novel glittering flowers.

A breeding line, 'MA21', has superior characteristics; early flowering, flexible stems, and appealing flower morphology. The line was selected from a segregating population of a cross between 'Nazareno' and 'MA20', a breeding line with dusky pinkish purple flowers with AVIs. *In vitro* plants of 'MA21' were irradiated by 320 MeV carbon ion beams of 15 Gy to produce color variations. After cutting back the irradiated plants three times, 400 individuals were tested in the field. Finally we have selected two individuals with glittering flowers, 'MA21Red' and 'MA21 Purple'. We confirmed the lack of AMalT transcripts by reverse transcription PCR in both varieties. The two selections have stable, glittering flowers in all petals after vegetative propagation to 500 individuals each. Both the red glitter and pinkish purple glitter varieties are highly novel in that they glitter more

than any other carnation cultivars and lines reported thus far (Figure 5).

Conclusions

We demonstrated that ion-beam breeding can alter and improve petal color and shading. We have succeeded in creating the most glittering carnation ever by taking advantage of new mutagenesis techniques combined with exploiting genomic information. The formula are, (i) selection of appropriate materials for ion-beam mutagenesis, (ii) creation of mutants from the materials by ion-beam irradiation, (iii) screening of AVIs individuals by AMalT marker, and (iv) improving cell shape to glitter. The strategy and formula developed here make it possible to breed glittering carnations with directed mutagenesis, and the procedures are applicable to other floricultural crops as well.

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