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Original Paper

Flower pigment mutations induced by heavy ion beam irradiation in an interspecific hybrid of *Torenia*

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Abstract The use of heavy ion beams is an effective method for inducing mutations in plants. After irradiation with beams of Nitrogen (N) or Neon (Ne), the frequency of flower color mutants increased through the regeneration of stem or leaf without lateral meristem in an interspecific hybrid of *Torenia*. From anthocyanin assay results, mutants were divided into two groups. One group involved the deletion of the blue gene (DFR). The other group involved the deletion or duplication of a gene related to pigment production. The results show that heavy ion beams are effective in obtaining artificial mutants.

Key words: Anthocyanin assays, heavy ion beams, *Torenia*.

The genus *Torenia* (*Scrophulariaceae*) includes 40 species, found in east, southeast and south Asia and Africa. *Torenia fournieri* is the only species used for breeding in horticulture. Varieties of *T. fournieri* are one of the best-known summer bedding plants throughout the world because of their heat tolerance. The varieties have some variations in flower color, but this range is not varied enough.

It is very important for the *Torenia* breeding program to expand their characteristics, for example, flower color, flower size, plant habit, plant size etc.

Heavy ion beam irradiation has often been reported as an efficient mutagenic method. Examples quoted in the literature include obtaining a new stable mutant of *Arabidopsis thaliana* with a spotted pigment in the seed coat (Tanaka et al. 1977), salt- and cobalt-tolerant plants (Abe et al. 2000), sterility in *Verbena hybrida* (Suzuki et al. 2002), variegation in *Petunia hybrida* (Miyazaki et al. 2001) and flower mutation in Dahlias (Hamatani et al. 2000). However, differences in their effectiveness have been found to depend on the apparatus (Tanaka et al. 1997) and on the sensitivity of various organs to ion beams (Abe et al. 1999).

This study involves creating mutants of the *Torenia* hybrid cv. 'Summer Wave' series (Suntory Flowers, Shiga, Japan) by heavy ion beam irradiation. The 'Summer Wave' series is an inter-specific hybrid variety, which is completely sterile, but it is impossible to expand their variation without artificial mutagensis. White and blue/white transgenic plants have been previously generated from 'Summer Wave Blue' (Suzuki et al. 2000).

Materials and methods

Plant materials

Leaf tissues and stem internodes without lateral meristems of the *Torenia* hybrid cv. 'Summer Wave Blue' (Suntory Flowers) were cultured in vitro on MS medium containing 30 g l^{-1} sucrose and 0.8% agar. A total of 25 plants were cultured per laboratory dish.

Irradiation method

The following heavy ions were used in this study at the RIKEN Accelerator Research Facility (RARF): ^{14}N and ^{20}Ne ions, supplied by a multi-stage accelerator system, consisting of a heavy-ion linac and an AVF cyclotron and a ring cyclotron. The range of the ion beams in water is 3.4 cm for 135 MeV nucleon^{-1} ^{14}N ions and 2.3 cm for 135 MeV nucleon−1 ^{20}Ne ions, respectively.

Irradiation treatments were conducted at a dosage range of 5, 10, 20 and 50 Gy for both ions; 40 leaves and

Abbreviations: AVF, Azimuthally Varying Field; DFR, Dihydroflavonol reductase; HPLC, High precision liquid chromatography; RARF, RIKEN Accelerator Research Facility.
This article can be found at http://www.jspcmb.jp/

five stem internodes were treated within each range.

Mutant isolation

After irradiation, plant materials were cultured on MS medium at 26°C and an 18-h light regime. When cell aggregate regeneration commenced, they were divided into four. After division, 10 shoots were cut from each adventitious shoot regenerated from the cell aggregate and acclimated in 8×16 cell trays. It was possible to select mutants by flower color after several weeks. Mutants had clear differences in flower color in comparison with control on observation.

Pigment analysis

Petal reflectance was quantified using a CM-2022 spectrophotometer (Minolta, Tokyo, Japan). Extraction, HPLC analysis and structural determination of anthocyanins were carried out as described previously (Fujiwara et al. 1997).

Results and discussion

Isolation of color mutation

Plants with floral color variations, induced by irradiation treatment of ^{14}N and ^{20}Ne ion, are shown in Figure 1. The flower of top left is control, second and third flowers are pale blue, fourth flowers is blue mutant in which is disappeared the deep blotches from both side of petal. The below left flower is pale pink, and second flower is pink mutant in Figure 1. The mutants which had color shade variations in pink and blue mutants in comparison with control were induced.

Table 1 shows the number of acclimated plants after irradiation and the frequency of flower color mutation. There was no significant difference in the number of acclimated plants and type of heavy ion or dosage, because adventitious bud regeneration is very high in *Torenia* and it is very easy to obtain a lot of buds. The frequency of floral color mutants per explants was significantly higher than no irradiation (NI). As the dosage of ^{14}N ion irradiation treatment increased, the frequency of mutants per explants and acclimated plants increased—the frequency was highest at 50 Gy (N50). Dosage with the ^{20}Ne ion was not related to the frequency of mutants. There was no significant difference in the frequency of mutants between ^{14}N ions and ^{20}Ne ions—the total number of floral color mutants in ^{20}Ne and ^{14}N ion irradiation treatments was 21 and 19, respectively.

Table 2 shows the relationship between number of floral color mutants and irradiation treatment. Blue mutants were observed at the 5- and 10-Gy irradiation treatment with ^{14}N ions (N5 and N10), while pale blue and pink mutants increased with increasing ^{14}N ion dosage. The same tendency was also observed in irradiation treatment with ^{20}Ne ions. A pale pink mutant was found at 50 Gy irradiation treatment with ^{14}N ion (N50). In control (NI), two mutants resulting from somatic mutations were obtained, one blue and the other pink. The blue-colored mutant was similar to blue mutant (17), the pink-colored mutant was similar to pink mutant (24) (Figure 2).

All mutants could be maintained the character of mutation by cutting, but some back mutations were

Figure 1. Flower features of *Torenia*. Control (left) and various mutants (right) are shown. Control, Pale blue, Pale blue and Blue mutant (top, from left), Pale pink and Pink mutant (below, from left).

Table 1. The number of acclimated materials after irradiation and the frequency of flower color mutation.

	Control	^{14}N ion irradiation treatment					^{20}Ne ion irradiation treatment					
Irradiation Gy	No irradiation	5	10	20	50	total	5	10	20	50	total	Treatment
Treatment name	NI	N5	N10	N20	N50		Ne5	Ne10	Ne20	Ne50		total
No. of explants	45	45	45	45	45	180	45	45	45	45	180	360
No. of acclimated materials	326	468	479	509	424	1880	475	461	428	533	1897	3777
Total No. of floral color mutant	2	4	4	5	8	21	5	4	7	3	19	40
Frequency per explants (%)	4.4	8.9	8.9	11.1	17.8	11.7	11.1	8.9	15.6	6.7	10.6	11.1
Frequency per acclimated plants (%)	0.61	0.85	0.84	0.98	1.89	1.12	1.05	0.87	1.64	0.56	1.00	1.06

Table 2. Relation between number of floral color mutants and irradiation treatment.

	Control	^{14}N ion irradiation treatment					^{20}Ne ion irradiation treatment					Treatment total
	NI	N5	N10	N20	N50	total	Ne5	Ne10	Ne20	Ne50	total	
Blue	1	1	2	0	0	3	3	4	0	0	7	11
Pale blue	0	2	1	1	5	9	1	0	5	1	7	16
Pink	1	0	1	4	2	7	1	0	2	2	5	13
Pale pink	0	0	0	0	1	1	0	0	0	0	0	1

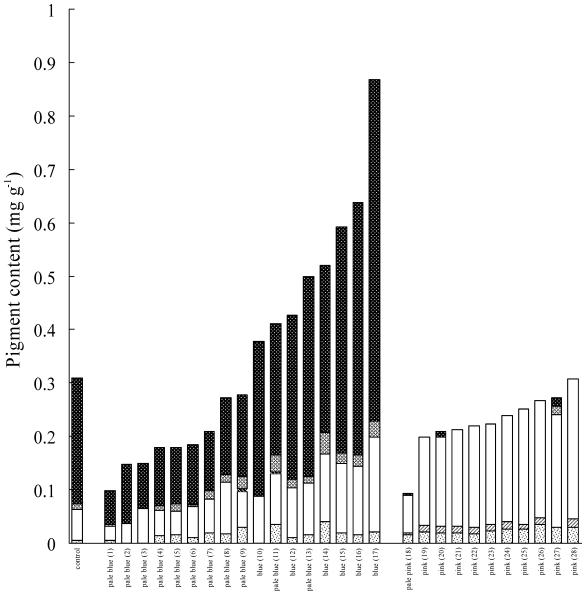

Figure 2. Pigment contents of control and mutants induced by irradiation of ^{14}N and ^{20}Ne ion. Pigments are malvidin (■), petunidin (▩), delphinidin (▨), peonidin (□), pelargonidin (▨) and cyanidin (▨).

observed on several shoots.

Analysis of flower pigment

The petal of *Torenia hybrid* cv. 'Summer Wave Blue' contained four anthocyanidins: malvidin (76.4%), peonidin (18.8%), petunidin (2.9%) and cyanidin (1.9%) (Figure 2). The mutants induced by ^{14}N and ^{20}Ne ion irradiation additionally contained pelargonidin and delphinidin. Mutants were classified into two groups by floral color: blue- and pink-colored mutants. Blue-colored mutants were distinguished by the differences of their nuance on observation. The concentration of floral pigment in blue-colored mutants was similar to the original plant, i.e. malvidin as the main pigment and additional amounts of peonidin, cyanidin and petunidin. Some mutants had small concentrations of delphinidin. Blue-colored mutants had variations in total amount of pigment—the most and least amount of pigment was 0.868 and 0.098 mg g^{-1}, respectively (Figure 2). Blue-colored mutants were also distinguished by pale blue and blue mutants, though they had continuous variation. Pale blue mutants, except for 11 and 13, had less total

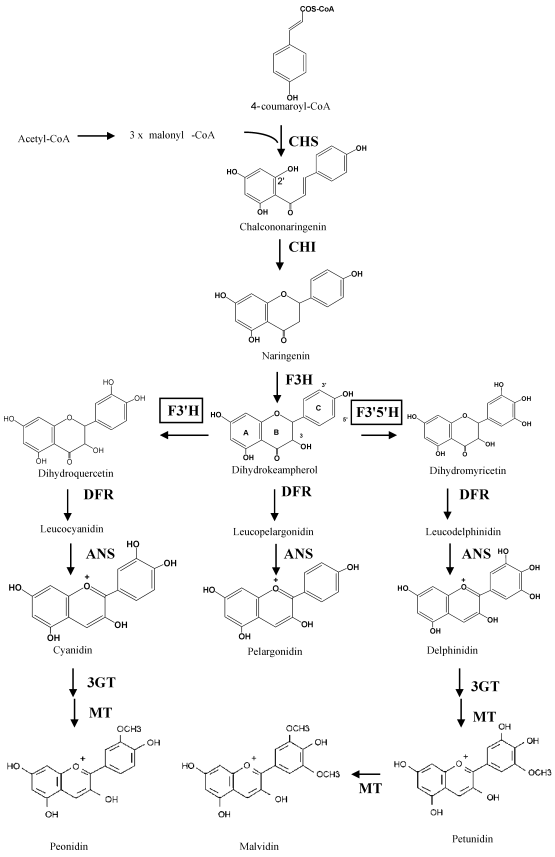

Figure 3. Anthocyanin biosynthesis pathway. CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3′H, flavonoid 3′-hydroxylase; F3′5′H, flavonoid 3′5′-hydroxylase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; 3GT, 3-glucosyltransferase.

pigment and malvidin content than control (original plant) (Figure 2), while blue mutants had more total pigment and malvidin content than control. Blue mutant 17, which had the highest total pigment content, contained a three times higher concentration of several pigments than control. Figure 3 shows the process of pigmentation (Suzuki et al. 2000). Because blue-colored mutants had no pelargonidin and floral pigment concentrations similar to the original plant, it was determined that blue-colored mutants were quantitative not qualitative mutants, i.e. pigment production was promoted in blue mutants and restrained in pale blue mutants.

The increase in total pigment in blue mutants is related to the changes in the pigment production gene expression, which was caused by the re-arrangement of DNA during adventitious bud regeneration after the gene was cut from the chromosome by the heavy ion beams. Although a decrease in color pigment often occurs with genetic transformation (Aida et al. 2000), an increase in color pigment is very unique. Therefore, the use of heavy ion beams was effective for acquirement of mutants in flower color induced by decreasing or increasing color pigment, and the mutants were beneficial for the horticultural industry.

Floral pigments in pink-colored mutants consisted mainly of peonidin, cyanidin and pelargonidin, in contrast to the original plant. Pink-colored mutants had little or no malvidin and petunidin, in contrast to blue-colored mutants and were characterized by containing significantly high concentrations of peonidin and cyanidin, in comparison with blue-colored mutants. The accumulation of peonidin and cyanidin in pink-colored mutants indicated that the expression of DFR, ANS, 3GT, 5GT and MT was not affected by heavy ion beam irradiation (Figure 3). We, therefore, decided that the loss of malvidin and petunidin in pink-colored mutants was induced by the inhibition of dihydromyricetin biosynthesis and the inhibition was induced by the re-arrangement of DNA for F3′5′H by heavy ion beam irradiation (Figure 3). The high content of peonidin and cyanidin and the new biosynthesis of pelargonidin were induced by blocking of the delphinidin pathway. This result is very similar to that obtained by cosuppression of anthocyanin biosynthesis genes (Suzuki et al. 2000).

Reflectance

Blue mutants had a wide range of reflectance between 0.2 and 28.7%, while the range of reflectance in pink mutants was narrow (4.8–9.3%), excepting a pale pink mutant. In pink mutants, all the values of reflectance was higher than control, but there was no relationship between anthocyanin content and reflectance (Figure 4). Blue mutants had low reflectance regardless of their anthocyanin content. Anthocyanin content in blue mutants was higher than those in pale blue mutants, and pale blue mutants indicated high reflectance. Pale blue mutants, therefore, could be distinguished from control and blue mutants.

Figure 5 shows the relationship between malvidin or peonidin content and reflectance in blue and pale blue mutants. As regards peonidin and reflectance, peonidin content in blue and pale blue mutants, as well as control was not different, in spite of the large difference in reflectance between blue and pale blue mutants. It was determined that peonidin did not contribute to reflectance. There was significant negative correlation of malvidin content with reflectance; reflectance in pale blue mutants, which had low malvidin content, was higher than those in blue mutants, which had high malvidin content (Figure 5). It was indicated that malvidin content influenced petal reflectance.

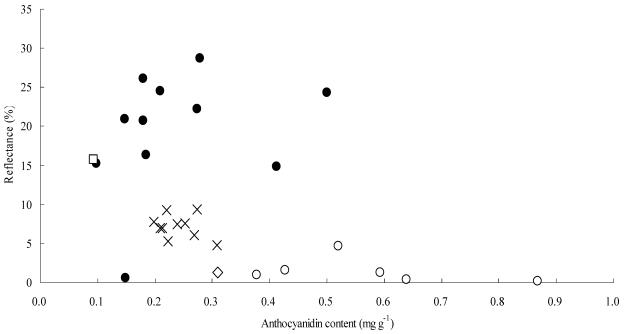

Figure 4. Relationship between Anthocyanin content and reflectance. Mutants are blue mutants (○), pale blue mutants (●), pink mutants (×), pale pink mutants (□) and control (◇).

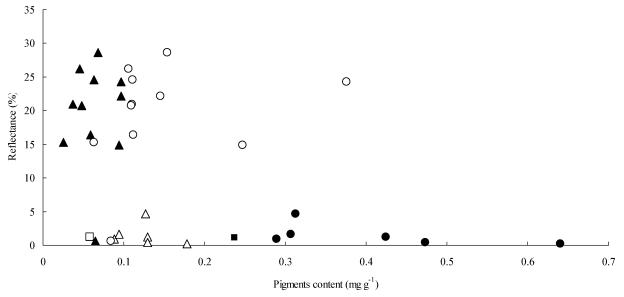

Figure 5. Relationship between peonidin or malvidin content and reflection. Pigments in mutant are peonidin content in pale blue mutants (▲), peonidin content in blue mutants (△), malvidin content in pale blue mutants (○), malvidin content in blue mutants (●), peonidin content in control (□) and malvidin content in control (■).

Flower color is one of the most important character for commercial values of flower, and flower color is influenced by light reflection and permeability, though the kind, quantity and assortment of pigment are basically major factor of flower color. Light reflection is affected not only by pigment, but also by cell structure (Qon et al. 1981). The shape, size and surface striations of petal epidermal cells affect light reflection (Hayashi et al. 2003). Differences in the reflectance for peonidin contents are considered to be the result of heavy ion beam irradiation causing changes in the structure of epidermal cells in *Torenia* petal.

Transgenic plants retain the characteristics of the host and only target characteristics are modified (Tanaka et al. 1998). Genetic engineering is effective in changing a target character, but mutations, which can be induced efficiently, as in this study, can be effective in expanding the variation of unspecified characters, especially for sterile plants, such as *Torenia* hybrid cv. ‘Summer Wave’.

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