

Technical Note

Efficient induction of flower-color mutants by ion beam irradiation in petunia seedlings treated with high sucrose concentration

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Abstract We examined the effect of pretreatment on the frequency of flower-color mutants induced by ion beams. We found that petunia seedlings treated with 3% sucrose from 8 days after sowing accumulated significant amount of pigments within 4 days compared to non-treated control seedlings. The petunia seedlings treated with sucrose were exposed to 320-MeV carbon ions. The sucrose treatment did not affect the survival rate and seed fertility of the M₁ plants. In the M₂ lines obtained by self-pollination of individual M₁ plants, chlorophyll mutants were obtained in both treated and non-treated groups with a similar frequency. Flower-color mutants that included magenta, purple, light pink and white were obtained from the original violet color. The frequency of flower-color mutants was significantly higher in the sucrose-treated group than in the non-treated group. These results suggest that sucrose pretreatment specifically increases the frequency of flower-color mutation following ion beam irradiation.

Key words: Flower color, ion beam, mutation, petunia, sucrose.

Ionizing radiation has been used for several decades to produce new plant varieties. During this period, many attempts have been made to increase the mutation efficiency. Efficient mutagenesis requires induction of desirable changes free from undesirable effects. Higher mutation frequency can be achieved by higher doses within the limits of survival. However, for practical plant breeding, the irradiation dose should be minimized to avoid the induction of undesirable mutations. It has been shown that external factors, such as oxygen concentration, water content and temperature enhance radiation sensitivity (van Harten 1998), in which case the irradiation dose must be reduced to avoid undesirable mutations. Therefore, it has been thought to be difficult to increase the efficiency of mutagenesis through sensitivity-modifying factors in practical plant breeding.

Nagatomi et al. (1997, 1998), who examined the mutagenic effects of carbon-ion beams in chrysanthemums, reported that the flower-color mutants were obtained with higher frequency when the cultured petals rather than the cultured leaves were irradiated. They hypothesized that the genes involved in flower-color synthesis are easily mutated where these genes are

highly expressed. If we could induce mutations on particular genes or chromosomal regions, it is very useful to increase the mutation efficiency. Since Nagatomi et al. (1997, 1998) compared the mutagenic effect of identical irradiation doses in petals and leaves, the difference of radiation sensitivity between the tissues was not considered. In addition, they did not examine mutations other than flower color as an internal standard for general mutation frequency. Therefore, it has not yet been clearly shown if the condition of the cells alters the mutation frequency of particular genes.

Gamma-rays and X-rays have long been used for mutation induction in plant breeding. In recent years, researchers have focused on ion beams as a new mutagen, and several studies have shown that the ion beams induce a wider mutation spectrum with higher mutation frequency as compared to gamma-rays or X-rays (Honda et al. 2006; Okamura et al. 2003, 2006; Sasaki et al. 2008; Shikazono et al. 2003). Okamura et al. (2003) reported that ion beams induced flower colors that were not obtained by gamma-rays. Flower color is one of the most important traits in horticultural plants. Expression of genes involved in anthocyanin pigment

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biosynthesis are known to be activated by stress, such as high-intensity light, UV light or low temperature (Dubos et al 2008; Hase et al. 2006; Olsen et al 2009). Sugars are also known to induce anthocyanin accumulation (Hara et al. 2003; Mita et al. 1997; Tsukaya et al. 1991). We hypothesize that if the condition of the cell could alter the mutation frequency of particular genes, pretreatment with those factors would likely increase the frequency of flower-color mutation. In this study, we examined the effect of pretreatment on the frequency of flower-color mutants in petunia. The frequency of chlorophyll mutants was examined as an internal standard for general mutation frequency.

The violet-flowered BBss11 line of petunia (*Petunia hybrida*), genetically fixed by self-pollination over 10 generations, was used for this study. This line has been used as a maternal parental line of the F1 hybrid petunia "Wave Blue" sold by Kirin Agribio Co., Ltd (Sakura, Tochigi, Japan). Spontaneous variation of flower color was not found in 10,000 plants grown under conventional cultivation. To find the suitable pretreatment conditions that stimulate pigment biosynthesis, we examined the effects of sucrose, UV light and low-temperature treatment in petunia seedlings. Seeds were sown in petri dishes filled with soil (Plugmate, Honenagri Co., Ltd, Niigata, Japan). The dishes were kept in a growth room at 23°C under a 16/8 h photoperiod with approximately 4,000 lux of white fluorescent light. The dishes were watered with distilled water to keep them continuously wet. For sucrose treatment, 1 ml of 20% sucrose solution was added 8 days after sowing. The final concentration of sucrose is estimated to be around 3%. For UV-light treatment, 8-day-old seedlings were exposed to a pulse of UV-B light (CSL-30B, COSMO BIO, Tokyo, Japan) at a dose of 15 kJ m⁻². For cold temperature treatment, 8-day-old seedlings were kept at 4°C for 24 or 48 hr. In the seedlings treated with 3% sucrose, increased pigment accumulation was observed at 2 days after the addition of sucrose, with a remarkable difference developing 4 days after the addition of sucrose (Figure 1). High accumulation of pigments was observed in the hypocotyls, shoot apices and basal part of the petioles. UV light also stimulated the pigment accumulation but was much less effective than the sucrose treatment. In addition, UV light caused damage to the leaves. The low-temperature treatment was not effective in the conditions tested. From these observations, treatment with sucrose for 2 days was thought to be suitable and enough to stimulate the genes involved in pigment accumulation in petunia seedlings.

In order to determine the appropriate dose to examine the effects of sucrose treatment on mutation frequency, 10-day-old seedlings grown in petri dishes as described above were exposed to 0 to 30 Gy of 320-MeV carbon ions (¹²C⁶⁺). We used the irradiation chamber connected

with a vertical beam line from an AVF cyclotron at the Japan Atomic Energy Agency (Takasaki, Gunma, Japan) (Tanaka et al. 1997a). The linear energy transfer of the carbon ions at the surface of the sample was calculated to be 76 keV/μm by the IRACM code (Tanaka et al. 1997b), with the water-equivalent range of the carbon ions calculated to be 2.2 mm. Since we aimed to examine mutations in the M₂ generation, the shoot apical meristem was the irradiation target. The 10-day-old seedlings had fully-opened cotyledons and the shoot apices were relatively exposed. The range of carbon ions is thought to be enough to penetrate the shoot apical meristem of the 10-day-old seedlings. One day after irradiation, the seedlings were transplanted to pots and grown in a green house at the Japan Atomic Energy Agency. When we transplanted the irradiated seedlings to pots, we noticed that the root elongation of sucrose-treated seedlings was highly suppressed compared to non-treated seedlings. The survival rate at 1 month after irradiation was more than 90% up to 10 Gy in both sucrose-treated and non-treated groups, although the growth of the plants was gradually suppressed as the dose increased. Almost no plants survived at doses more than 20 Gy. It was thought that the sucrose treatment did not affect the mortality of the seedlings. For mutagenesis by ion beams, our experience has shown that it is better to choose a dose that does not decrease the survival rate so as to reduce undesirable mutations. Therefore, we determined 8 Gy to be an appropriate dose for mutagenesis in this experiment.

For mutant screening, the petunia seedlings treated with or without sucrose were exposed to 8 Gy of carbon ions, and the M₂ seeds were collected from individual M₁ plants. Plants were grown in a green house at Kirin Agribio or Japan Atomic Energy Agency. The seeds of M₂ lines were allowed to germinate on the soil, and the frequency of chlorophyll mutations was determined. Around 5 to 20 seeds, in most cases more than 10 seeds, were sown for each M₂ line. The germinated seedlings were grown in a green house, and the frequency of flower-color mutants was determined. Mutant screening was carried out in three independent experiments. The results are summarized in Table 1. In Experiment 1, M₂ seeds were collected from less than 20% of M₁ plants. This is most likely because the growth pots used were small. In Experiment 2, M₂ seeds were collected from approximately half of M₁ plants. For un-irradiated control plants in Experiment 2, progeny seeds were collected from 83% of the plants. This suggests that the seed fertility of M₁ plants was partially decreased by the ion-beam irradiation. In Experiment 3, we grew M₁ plants in larger pots, and M₂ seeds were collected from more than 90% of M₁ plants.

In the M₂ generation, chlorophyll mutants were obtained in both sucrose-treated and non-treated groups.

The frequencies of chlorophyll mutants were 0.56%, 0.39% and 0.55% in the sucrose-treated group and 0.48%, 0.33% and 0.43% in the non-treated group. There was no significant difference between the two groups in each of the three independent experiments. No chlorophyll mutant was obtained in the un-irradiated control group of Experiment 2. The three M_2 lines of chlorophyll mutants in Experiment 1 segregated into ratios of 4 mutants/16 total plants examined (=25%), 1/7 (=14%) and 3/20 (=15%). This suggests that these chlorophyll mutations are single recessive. The number of germline cells in 10-day-old petunia seedlings is expected to be one or two. Flower-color mutants that included magenta, purple, light pink and white were obtained from the original violet color (Figure 2). Magenta and purple were the most frequent color variations obtained in this study. The frequencies of flower-color mutants were 1.52%, 1.20% and 1.26% in the sucrose-treated group and 0.56%, 0.58%, and 0.47% in the non-treated group. The sucrose treatment resulted in a more than two-fold increase in flower-color mutant frequency in the three

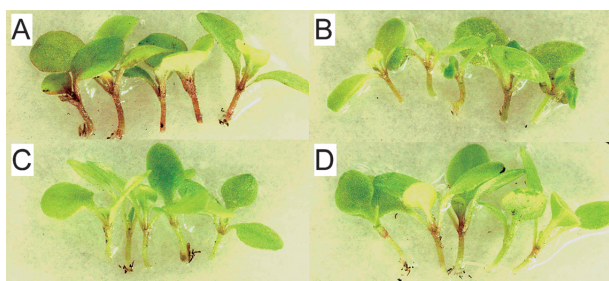


Figure 1. Effect of pretreatments on accumulation of pigments in petunia seedlings. Eight-day-old seedlings were treated with 3% sucrose (A), a pulse 15 kJ m^{-2} of UV-light (B) or 4°C (C) for 24 hour. Seedlings at 4 days after treatment are shown. The control plants are shown in (D).

independent experiments. These results show that sucrose pretreatment of petunia seedlings significantly increased the frequency of flower-color mutants without changing the frequency of chlorophyll mutants following ion-beam irradiation.

Our results suggest that the sucrose treatment specifically affected the mutation frequency of genes involved in the expression of flower color. If this method is also applicable for other ornamental plants, it is expected to improve the efficiency of practical mutation breeding for flower color. In general, all mutations are expected to occur randomly over the entire genome. Although the mechanism underlying the increased mutation frequency observed here is presently unclear, it is thought that sucrose pretreatment increases the radiation sensitivity of particular genes or chromosomal regions concerning pigment biosynthesis. It has been experimentally shown by measurement of DNA fragmentation using pulsed-field gel electrophoresis in mammalian cell lines that DNA double-strand breaks (DSBs) are induced with high frequency in de-condensed chromatin (Elia and Bradley 1992; Newman *et al.* 2000; Radulescu *et al.* 2004). Falk *et al.* (2008), who directly visualized gamma-ray-induced DSBs in human cells by immuno-fluorescent in situ hybridization, showed that the condensed chromatin in gene-poor regions is much less susceptible to DSB induction than de-condensed chromatin with highly expressed genes. These studies suggest that the mutation frequency of genes in particular regions of chromosomes could be affected by the packaging status of chromatin. A similar mechanism might be responsible for the specific increase of flower color mutations observed here.

In our experiment, the petunia seedlings were under high-sucrose concentration for 3 or 4 days, and only

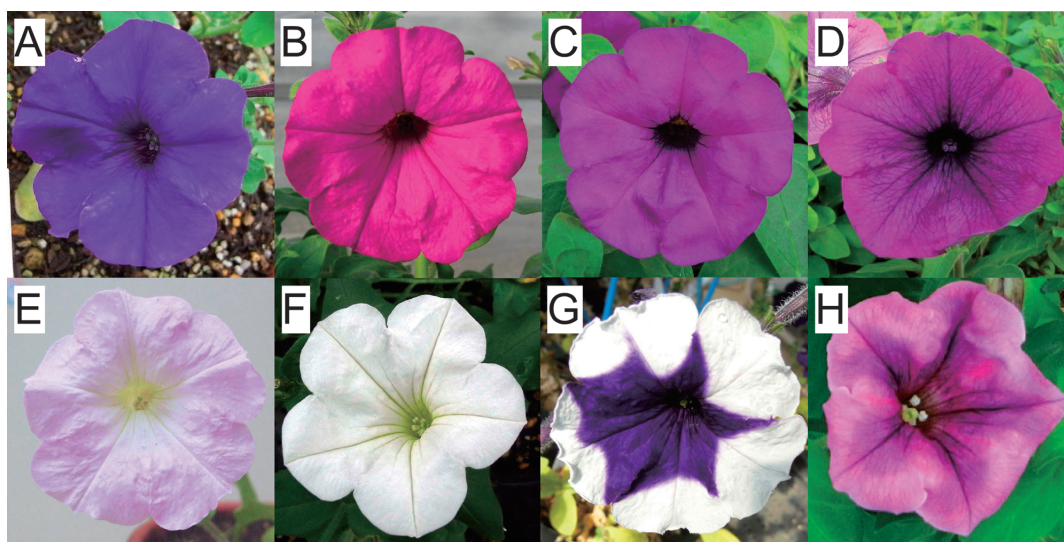


Figure 2. Parental line of petunia and flower-color mutants obtained in this experiment. (A) Parental line with violet flower color. (B–H) Flower-color mutants. (B) Magenta, (C) Purple, (D) Purple vein, (E) Light pink, (F) White, (G) Blue picotee, (H) Burgundy.

Table 1 Effect of pretreatment with sucrose on the frequency of chlorophyll and flower-color mutation

Experiment	Dose of carbon ions (Gy)	Addition of sucrose	Number of M ₁ plants	Chlorophyll mutants		Flower-color mutants		Flower color and number of M ₂ lines
				Number of M ₂ lines investigated for chlorophyll mutants	Number of M ₂ lines contained chlorophyll mutants	Number of M ₂ lines investigated for flower-color mutants	Number of M ₂ lines contained flower-color mutants	
Experiment 1	8	+	1,900	354	2 (0.56%)	329	5 (1.52%)*	Magenta (3), Light purple (1), Light pink (1)
	8	-	1,900	210	1 (0.48%)	177	1 (0.56%)	Red purple (1)
Experiment 2	8	+	1,900	1,018	4 (0.39%)	1,003	12 (1.20%)**	Purple (4), Magenta (4), Purple vein (2), Light pink (1), White (1)
	8	-	1,900	909	3 (0.33%)	868	5 (0.58%)	Purple (3), Magenta (2)
	0	-	256	212	0 (0.00%)	Not tested	Not tested	Not tested
Experiment 3	8	+	2,176	2,001	11 (0.55%)	1,977	25 (1.26%***)	Purple (6), Magenta (5), Purple vein (3), Light pink (3), Blue picotee (2), Magenta picotee (2), White (2), Burgundy (1), Light blue (1)
	8	-	2,176	2,099	9 (0.43%)	1,915	9 (0.47%)	Purple (3), Magenta (3), Blue (2), Light pink (1)

* ** ** Statistically higher from corresponding control value predicted by Poisson distribution (* p = 0.029, ** p = 0.009, *** p < 0.001).

aboveground parts of the seedlings were irradiated with carbon ions. Root elongation was highly suppressed during this period. This might be due to a rapid change of osmotic pressure. The suppression of root growth is thought to be a transient effect because the survival rate and seed fertility were not different between sucrose-treated and non-treated groups. Hara et al. (2003) reported that detached radish hypocotyls treated with 175 mM sucrose accumulated a greater amount of anthocyanin. Stimulation of anthocyanin accumulation was also observed in detached Arabidopsis leaves treated with 6% sucrose solution (Mita et al. 1997). Since mannose and glucose analog did not elevate anthocyanin content in radish hypocotyls, Hara et al. (2003) concluded that the effect of sucrose on anthocyanin accumulation was not an osmotic response. Further experiments are needed to elucidate the physiological action of sucrose in petunia seedlings.

We found that sucrose-treated seedlings accumulated much greater pigment amounts than non-treated seedlings. Accumulation of pigments is closely associated with expression of genes involved in pigment biosynthesis. It is thought that the genes concerning pigment biosynthesis are activated in sucrose-treated petunia seedlings. Similarly, increased expression levels of anthocyanin-related genes were reported in radish hypocotyls treated with sucrose (Hara et al. 2004). Petunia flower color is essentially governed by anthocyanin pigmentation and vacuole pH values (Grlesbach 1996). Genetic analysis has shown that a number of genes are involved in biosynthesis and color expression of anthocyanins. Flower color is expected to change when the activity of just one of those genes is altered by mutation. It is thought that the set of genes expressed in seedlings partially differs from the genes expressed in flowers. For example, 12 isozymes of chalcone synthase (CHS) have been cloned in petunia, but among them, only CHS-A and CHS-J are shown to be expressed in flower coloration (Koes et al. 1989). It is also known that regulation of anthocyanin biosynthesis is primarily achieved by transcriptional regulation of biosynthetic genes. The Mitchell line of petunia, which has a white flower due to mutations in two anthocyanin-regulatory genes, accumulates small amount of anthocyanins in the leaves and stems when the plants were grown in high-light conditions (Albert et al. 2009). Therefore, the regulation of gene expression induced by exogenous factors in vegetative tissues could be different from the regulation of gene expression in flowers. An explanation for the phenomenon remains obscure, and further experiments are necessary to elucidate the underlying mechanism and determine which genes are responsible for flower-color changes observed here. However, our results clearly show the possibility that mutagenesis can be regulated to a greater degree than

previously thought.

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