

## Short Communication

## Biological effects of heavy-ion beam irradiation on cyclamen

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**Abstract** The biological effects of heavy-ion beam irradiation on cultured tissues of cyclamen were investigated by establishing an irradiation-mediated mutation breeding protocol for producing a new variety of cyclamen. Initially, a callus, a somatic embryo, and a plantlet were irradiated with <sup>12</sup>C<sup>6+</sup> ion beam at doses of 10, 20, 40, 60, and 80 Gy. Distinct mutants were not obtained in plants regenerated from the irradiated cultured materials. Next, we used the tuber of cyclamen as a target for irradiation. Irradiation (8–16 Gy) of 8–15 mm diameter tubers produced male sterility, change in petal colour, and petal form. Mutation induction by heavy-ion beam irradiation to the tuber is useful for changing flower characteristics of cyclamen.

**Key words:** Cyclamen, ion beam, tuber.

*Cyclamen* is a plant genus within the family *Primulaceae* comprised of 22 species (Grey-Wilson 2002). The plants grow wild in parts of Europe, western Asia, and parts of North Africa. *C. persicum*, particularly, is used as a breeding material and many ornamental varieties with various characteristics have been developed with this species (Tsukamoto 1994). Cyclamen is a popular winter plant, with about 22 million pots produced every year in Japan alone. As with other plant varieties, new cyclamen varieties are typically produced in a time-consuming process of repeated crossing and phenotypic selection. The ability to hasten this process would be desirable.

Mutation breeding can induce a drastic change in the target organism and shorten the period required for breeding new varieties. Mutations are created by irradiation, gamma rays, X-rays, and neutrons have been used mainly for mutation breeding. Recently, heavy-ion beam irradiation has attracted increasing attention as a new mutation method. Heavy-ion beam irradiation is a type of high linear energy transfer (LET) radiation, which bombards the target with higher energy that can be achieved using low LET forms of radiation such as gamma rays, X-rays, and neutrons. Heavy-ion beam irradiation induces plant mutants with high frequency, shows broad mutation spectrum, and produces novel mutants (Tanaka 1999). Polymerase chain reaction and Southern blot-based analyses of the effect of heavy-

ion beam irradiation on *Arabidopsis thaliana* has demonstrated the induction of various types of mutant alleles (Shikazono et al. 1998); the resulting novel mutants include ultraviolet-B resistant mutant (Tanaka et al. 2002), anthocyanin spotted testa mutant (Tanaka et al. 1997), and serrated petal mutant (Hase et al. 2000). In *Nicotiana tabacum*, mutants tolerant to herbicide, salt, and cobalt (Abe et al. 2000) and Potato Virus Y (Hamada et al. 1999) have been produced. Other examples from ornamental plants include a sterile mutant of *Verbena hybrida* (Suzuki et al. 2002), a variegated mutant of *Petunia hybrida* (Miyazaki et al. 2002), and flower colour mutant of dahlia, rose, hybrids of *Torenia*, chrysanthemum, and carnation (Hamatani et al. 2001; Hara et al. 2003; Yamaguchi et al. 2003; Miyazaki et al. 2006; Nagatomi et al. 1997; Okamura et al. 2003). Some of these mutants have been commercialized.

To our knowledge, there are no reports on the development of new cyclamen varieties using heavy-ion beam irradiation. The present study was undertaken to establish an efficient heavy-ion beam irradiation protocol for producing new varieties of cyclamen. We examined the effect of the beam on cyclamen callus, somatic embryo, plantlet, and tuber.

*C. persicum* cv. 'Fragrance Mini' (FM) and 'Wink' (WK) cultivated in a green house under natural daylight conditions were used. FM is capable of very high

somatic embryo production and WK can copiously produce adventitious buds (Terakawa et al. 2008).

The effect of  $^{12}\text{C}^{6+}$  ion beam irradiation on somatic embryogenesis from a callus was examined using scapes of FM obtained from potted plants. Each scape was briefly washed under running tap water. The rinsed explants were surface sterilized with 70% (v/v) ethanol for 10 s and with 0.5% (v/v) sodium hypochlorite for 20 min. The explants were then rinsed three times with sterilized water and were cut into segments approximately 5 mm in length. The segments were placed on Murashige & Skoog (MS) medium (pH 5.8) (Murashige and Skoog 1962) containing  $0.1\text{ mg l}^{-1}$  kinetin,  $4\text{ mg l}^{-1}$  2,4-dichlorophenoxy acetic acid, 5% (w/v) sucrose, and 0.3% gellan gum. Callus production occurred during the subsequent month-long incubation at 25°C in the dark. Fifteen calli selected from the culture were exposed to a  $^{12}\text{C}^{6+}$  ion beam (1.62 GeV, LET 23  $\text{keV } \mu\text{m}^{-1}$ ) at the RIKEN Nishina Center, RI-Beam Factory at doses of 10, 20, 40, 60, or 80 Gy. After irradiation, calli were transferred to fresh medium and cultured under the aforementioned conditions for three months. The fresh weight of the somatic embryo produced on the callus was then determined.

Figure 1A depicts the results of the experiment. Higher irradiation produced progressively more severe suppression of somatic embryogenesis. Only 0.2 g of somatic embryo was produced at the highest dose of 80 Gy, while non-irradiated controls produced 1.1 g of somatic embryo.

The effect of  $^{12}\text{C}^{6+}$  ion beam irradiation on the survival rate of somatic embryo was examined as follows. Somatic embryos were produced by culturing sterile scape segments of FM as described above. Somatic embryos ( $n=210$ ) were irradiated using the aforementioned doses. The irradiated and non-irradiated (control) somatic embryos were transferred onto fresh medium and cultured as described in the first experiment for two months. The survival rate of somatic embryo was then examined. Survivors were considered to be embryos on which secondary embryos were formed. The survival rate decreased with increasing dose (Figure 1B). Only 11% of the embryos irradiated at 60 and 80 Gy survived, while 41% of the control somatic embryos survived.

The effect of  $^{12}\text{C}^{6+}$  ion beam irradiation on the plantlet survival rate was examined as follows. A plantlet was prepared by germinating FM somatic embryo; embryos were placed on MS medium (pH 5.8) containing  $0.1\text{ mg l}^{-1}$  6-benzylaminopurine (BAP),  $0.01\text{ mg l}^{-1}$   $\alpha$ -naphthylacetic acid (NAA),  $0.2\text{ mg l}^{-1}$  gibberellic acid  $A_3$ , 3% (w/v) sucrose, and 0.5% gellan gum. Plantlets developed during a one-month period at 20°C under fluorescent illumination (3000 lux) with a 16 h photoperiod. Seventy plantlets were irradiated as described above. After irradiation, plantlets were

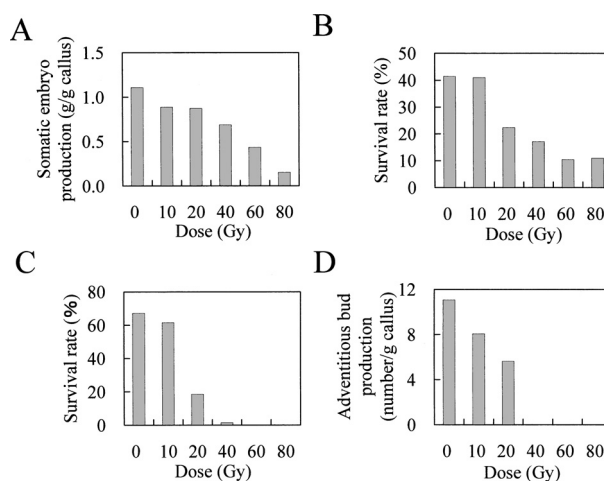


Figure 1. Effect of  $^{12}\text{C}^{6+}$  ion beam irradiation on (A) somatic embryogenesis, (B) survival rate of somatic embryos, (C) survival rate of plantlets, and (D) adventitious bud production from *C. persicum* calli. *C. persicum* cv. 'Fragrance Mini' (A, B, C) and 'Wink' (D) were used for irradiation material.

individually transferred to fresh medium and maintained as described above for three months. The survival rate after that time is summarized in Figure 1C. All plantlets irradiated at doses of 60 Gy and higher died while 67% of the control plantlets survived, indicating that plantlets are more susceptible to heavy-ion beam irradiation than somatic embryos.

The effect of  $^{12}\text{C}^{6+}$  ion beam irradiation on adventitious bud production from a callus was examined as follows. Calli were obtained from WK leaves. The leaves were excised from the potted plant and surface sterilized. After rinsing with sterilized water, the leaves were cut into squares of approximately 10 mm. These were placed on one-third-strength MS medium (pH 5.8) containing one-third strength MS salts,  $1\text{ mg l}^{-1}$  BAP,  $0.1\text{ mg l}^{-1}$  NAA, 3% (w/v) sucrose, and 0.35% gellan gum, and were cultured at 20°C in the dark for two months. They were then transferred to the medium modified with respect to the concentrations of BAP ( $0.2\text{ mg l}^{-1}$ ) and NAA ( $0.02\text{ mg l}^{-1}$ ) and cultured for four months. Callus production occurred during that time. Calli (16 g) were irradiated as described above and were then transferred to fresh medium and cultured under the aforementioned conditions. Adventitious bud production was then enumerated. As shown in Figure 1D, bud production decreased in a dose-dependent manner, with no bud production observed in calli irradiated at a dose of 40 Gy or higher.

Based on the data from these experiments, we attempted to obtain a cyclamen mutant by heavy-ion beam irradiation of calli, somatic embryos, and plantlets. These efforts were unsuccessful. Next, we decided to irradiate tubers of FM cultivars. The tubers were irradiated at doses of 4, 8, 12, and 16 Gy to examine the dose effect on cultured tuber viability. To prepare tubers,

Table 1. Effect of  $^{12}\text{C}^{6+}$  ion beam irradiation on survival and mutation of *C. persicum* cv. 'Fragrance Mini'

Dose (Gy)	Number of plants surviving <sup>1</sup>	Survival rate (%)	Male sterile mutants		Petal color mutants		Petal form mutants	
			No.	(%) <sup>2</sup>	No.	(%)	No.	(%)
Control	27	90	0	(0)	0	(0)	0	(0)
4	20	67	0	(0)	0	(0)	0	(0)
8	26	87	1	(3.8)	0	(3.8)	0	(3.8)
12	15	50	2	(13.3)	2	(13.3)	2	(13.3)
16	19	63	1	(5.3)	0	(5.3)	0	(5.3)

<sup>1</sup> 30 specimens were submitted for each dose

<sup>2</sup> Frequency of mutants (%)=(Number of mutants/Number of plants surviving)×100

plantlets were germinated from somatic embryos as detailed earlier in the text and were placed on hormone-free, one-third strength MS medium (pH 5.8) containing 3% (w/v) sucrose and 0.35% gellan gum, and placed at 20°C under fluorescent light (3000 lux) with a 16 h photoperiod for three months. The developed plants, whose tuber diameters ranged from 8–15 mm, were selected, and their leaves and roots were removed. The tubers were irradiated (30 specimens per dose), transferred onto fresh medium, and cultured at 20°C under fluorescent light (3000 lux) with a 16 h photoperiod for three months for plant elongation and rooting. Plants were transferred to flowerpots and were grown in a greenhouse under natural daylight conditions. Ten months later, the survival of the plants relative to the total number of irradiated tubers and the mutations in flower morphology were examined.

The effects of the irradiation on the plants are shown in Table 1. The survival rate ranged from 50–90%. The growth of irradiated specimens at 12 and 16 Gy was slightly arrested just after the irradiation, but recovered later. Male sterile mutants whose anthers were atrophic or deficient were obtained at an irradiation dose of 8–16 Gy (Figures 2B, 2C). Two petal colour mutants were obtained at 12 Gy. The petals of one mutant were reddish purple, instead of the original bluish purple, and those of the other mutant displayed a striped pattern of white over the underlying original colour (Figure 3B). Two petal forming mutants were also obtained at 12 Gy (Figure 3C). All the mutated plants formed both mutated and normal flowers, indicative of the chimeric nature of the mutations.

The present results indicate that mutation induction by heavy-ion beam irradiation to the tuber is useful for changing flower characteristics in a shorter time than is possible through conventional breeding. Further experiments are needed to evaluate the stability of the mutations. We are now regenerating plants from the petals and scapes of the flower mutant by the tissue culture method.

In this study, male sterile mutants incapable of pollen production were obtained. Recently, pollen-mediated gene flow from transgenic plants to wild relatives has

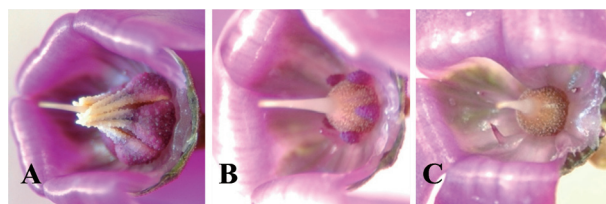


Figure 2. Photograph of typical male sterile mutant of *C. persicum* cv. 'Fragrance Mini' obtained by  $^{12}\text{C}^{6+}$  ion beam irradiation (8–16 Gy) of tubers. (A) Non-irradiated wild type, (B) anther atrophic mutant, and (C) anther deficient mutant.



Figure 3. Photograph of a typical petal mutant of *C. persicum* cv. 'Fragrance Mini' obtained by  $^{12}\text{C}^{6+}$  ion beam irradiation (12 Gy) of tubers. (A) Non-irradiated wild type, (B) petal colour mutant (striped pattern), and (C) petal form mutant.

become an important concern. Plants that lack pollen cannot disseminate their gene into natural environments via pollination. We produced some transgenic plants of cyclamen and the development of a male sterile mutant from these transgenic cyclamen by this method is in progress.

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### References

- Abe T, Bae CH, Ozaki T, Wang JM, Yoshida S (2000) Stress-tolerant mutants induced by heavy-ion beams. *Gamma Field Sym* 39: 45–56
- Grey-Wilson C (2002) *Cyclamen*. B T Batsford, London
- Hamada K, Inoue M, Tanaka A, Watanabe H (1999) Potato Virus Y-resistant mutation induced by the combination treatment of ion

- beam exposure and anther culture in *Nicotiana tabacum* L. *Plant Biotechnol* 16: 285–289
- Hamatani M, Iitsuka Y, Abe T, Miyoshi K, Yamamoto M, Yoshida S (2001) Mutant flowers of dahlia (*Dahlia pinnata* Cav.) induced by heavy-ion beams. *RIKEN Accel Prog Rep* 34: 169
- Hara Y, Abe T, Sakamoto K, Miyazawa Y, Yoshida S (2003) Effects of heavy-ion beam irradiation in rose (Rosa Hybrid cv. 'Bridal Fantasy'). *RIKEN Accel Prog Rep* 36: 135
- Hase Y, Tanaka A, Baba T, Watanabe H (2000) *FRL1* is required for petal and sepal development in *Arabidopsis*. *Plant J* 24: 21–32
- Miyazaki K, Suzuki K, Abe T, Katsumoto Y, Yoshida S, Kusumi T (2002) Isolation of variegated mutants of *Petunia hybrida* using heavy-ion beam irradiation. *RIKEN Accel Prog Rep* 35: 130
- Miyazaki K, Suzuki K, Iwaki K, Kusumi T, Abe T, Yoshida S, Fukui H (2006) Flower Pigment mutations induced by heavy ion beam irradiation in an inter specific hybrid of *Torenia*. *Plant Biotechnol* 23: 163–167
- Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant* 15: 473–497
- Nagatomi S, Tanaka A, Kato A, Watanabe H, Tano S (1997) Mutation induction on chrysanthemum plants regenerated from *in vitro* cultured explants irradiated with  $^{12}\text{C}^{5+}$  ion beam. *TIARA Ann Rep* 1995: 50–51
- Okamura M, Yasuno N, Ohtsuka M, Tanaka A, Shikazono N, Hase Y (2003) Wide variety of flower-color and -shape mutants regenerated from leaf cultures irradiated with ion beams. *Nucl Instr and Meth in Phys Res B* 206: 574–578
- Shikazono N, Yokota Y, Tanaka A, Watanabe H, Tano S (1998) Molecular analysis of carbon ion-induced mutations in *Arabidopsis thaliana*. *Genes Genet Syst* 73: 173–179
- Suzuki K, Yomo Y, Abe T, Katsumoto Y, Miyazaki K, Yoshida S, Kusumi T (2002) Isolation of sterile mutants of *Verbena hybrida* using heavy-ion beam irradiation. *RIKEN Accel Prog Rep* 35: 129
- Tanaka A, Tano S, Chantes T, Yokota Y, Shikazono N, Watanabe H (1997) A new *Arabidopsis* mutant induced by ion beams affects flavonoid synthesis with spotted pigmentation in testa. *Genes Genet Syst* 72: 141–148
- Tanaka A (1999) Mutation induction by ion beams in *Arabidopsis*. *Gamma Field Sym* 38: 19–27
- Tanaka A, Sakamoto A, Ishigaki Y, Nikaido O, Sun G, Hase Y, Shikazono N, Tano S, Watanabe H (2002) An ultraviolet-B-resistant mutant with enhanced DNA repair in *Arabidopsis*. *Plant Physiol* 129: 64–71
- Terakawa T, Yamamura T, Murayama T (2008) Improvement of Regeneration and Transformation System for *Cyclamen persicum* using Somatic Embryo Culture. *Plant Biotechnol* 77–80
- Tsukamoto Y (1994) *The grand dictionary of horticulture (Engei Shokubutsu Daijiten)*. Shogakukan, Tokyo (in Japanese)
- Yamaguchi H, Nagatomi S, Morishita T, Degi K, Tanaka A, Shikazono N, Hase Y (2003) Mutation induced with ion beam irradiation in rose. *Nucl Instr Meth Phys Res B* 206: 561–564