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Short Communication

Red-purple flower due to delphinidin 3,5-diglucoside, a novel pigment for *Cyclamen* spp., generated by ion-beam irradiation

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Abstract We previously bred fragrant cyclamen cultivars by interspecific hybridization between cultivars of *Cyclamen persicum* and the wild species *Cyclamen purpurascens*. One of these fragrant cultivars, Kaori-no-mai, blooms purple flowers containing malvidin 3,5-diglucoside as the major anthocyanin. Here, we irradiated etiolated petioles of Kaori-no-mai with a 320-MeV carbon-ion beam at 0–16 Gy to increase flower color variation by mutation. Some of the M_2 plants derived from self-pollination of M_1 plants irradiated at 2 Gy were flower-color mutants that retained desirable flower shape, flower size, and leaf color. One of the mutants bloomed novel red-purple flowers, the major anthocyanin of which was delphinidin 3,5-diglucoside. Loss of methylation activity at the anthocyanin 3′- and 5′-hydroxyl groups with little influence on anthocyanin concentration was attributed to the mutation. Because the major anthocyanins in flowers of *Cyclamen* spp. were previously restricted to malvidin, peonidin, and cyanidin types, the generation of a cyclamen containing mostly the delphinidin-type anthocyanin is an important breakthrough in cyclamen breeding. We expect this mutant to become not only a commercial cultivar itself, but also a valuable genetic resource for cyclamen breeding.

Key words: Anthocyanin, cyclamen, ion-beam, mutation.

Ornamental cyclamen cultivars have been developed through the selection of natural mutants of *Cyclamen persicum* ($2n=2x=48$) and crossing of the mutants (Grey-Wilson 2002). The flowers of these cultivars derived from only *C. persicum* are unperfumed. In earlier work (Ishizaka and Uematsu 1995a; b; Ishizaka et al. 2002), to increase their commercial value we introduced fragrance properties into cyclamen cultivars from another *Cyclamen* species, *C. purpurascens* ($2n=2x=34$), which has rose, hyacinth, and lily of the valley like fragrance. Conventional interspecific hybridization between cultivars of *C. persicum* and *C. purpurascens* originally failed owing to a cross-sterility barrier caused by abortion of the hybrid embryos. We overcame the barrier by rescuing weak hybrid embryos by means of tissue culture, and produced sterile hybrids ($2n=2x=41$) from the rescued embryos (Ishizaka and Uematsu 1995a). Furthermore, we successfully produced fertile amphidiploids ($2n=4x=82$) by polyploidization using colchicine treatment (Ishizaka and Uematsu 1995b). By crossing strains of the amphidiploid plants, we bred three cyclamen cultivars—Kokou-no-kaori, Uruwashi-no-kaori, and Kaori-no-mai—of *C. persicum*×*C. purpurascens* that emitted fragrance similar to *C. purpurascens* (Ishizaka 2008). The petal coloration of these fragrant cyclamens was purple or pink. We tried to increase coloration variety in order to much enhance ornamental value of the fragrant cyclamen.

For mutation breeding, gamma-ray or X-ray irradiation techniques are often used to improve a target trait while retaining other traits (Yamaguchi 2001). Ion-beam irradiation technique has recently been adopted for mutation breeding because it produces a broad mutation spectrum and furthermore tends to generate mutants affecting only one or few traits (Tanaka 1999; 2003).

Abbreviations: 1/2-N MS, Murashige and Skoog medium (Murashige and Skoog 1962) containing half the standard concentration of nitrogen sources; AR, 1/2-N MS containing 0.1 mg L^{-1} 1-naphthylacetic acid, 3% sucrose, and 3 g L^{-1} gellan gum and adjusted to pH 5.8; AS, 1/2-N MS containing 1 mg L^{-1} 2,4-dichlorophenoxyacetic acid, 0.5 mg L^{-1} 6-benzylaminopurine, 9% sucrose, and 3 g L^{-1} gellan gum and adjusted to pH 5.8; ESIMS, electrospray ionization mass spectrometry; HPLC, high-performance liquid chromatography; SG, 1/2-N MS containing 3% sucrose and 3 g L^{-1} gellan gum and adjusted to pH 5.8; TLC, thin layer chromatography

This article can be found at http://www.jspcmb.jp/

This technique has been used to produce several commercial cultivars of chrysanthemum (Nagatomi et al. 1997; Ueno et al. 2002), carnation (Okamura et al. 2003), osteospermum (Iizuka et al. 2008), dahlia (Hamatani et al. 2001), and verbena (Kanaya et al. 2008). In the present study, we performed ion-beam irradiation to induce coloration-specific mutants of Kaori-no-mai while retaining other desirable properties of fragrance, plant shape, and size.

Kaori-no-mai has purple petals composed of a basal deep purple 'eye' and the other major tissue 'slip' (Figure 1C). Seeds of Kaori-no-mai were surface-sterilized with 70% ethyl alcohol and 1% sodium hypochlorite, placed in a test tube containing SG medium under aseptic conditions, and held at 25°C in the dark. After the seeds germinated, the seedlings were kept under the same conditions to induce etiolated petioles. Etiolated petioles were excised approximately 3 mm in length when they reached about 50 mm in length and were placed in a 60-mm plastic Petri dish containing AS medium. The Petri dishes were covered with 30-H Kapton film (Du Pont-Toray Co., Ltd. Tokyo, Japan) and were irradiated with a 320-MeV carbon-ion beam accelerated by a TIARA AVF cyclotron (JAEA, Takasaki, Japan), at doses ranging from 0 to 16 Gy (Table 1). The irradiated etiolated petioles were

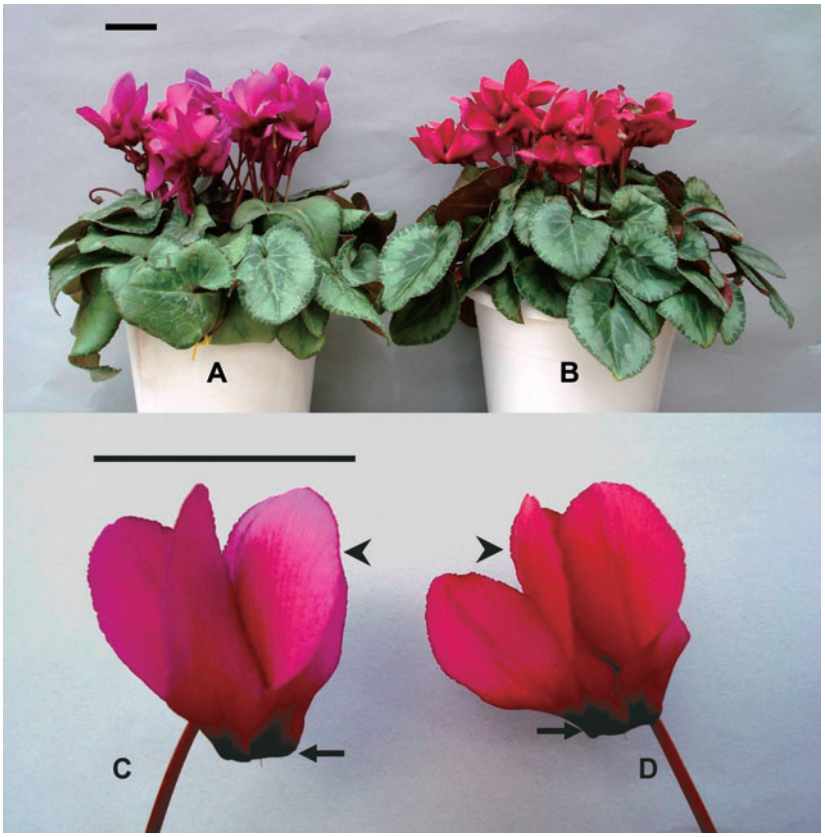

Figure 1. Whole cyclamen plants (A and B) and flowers (C and D) of purple-flowered 'Kaori-no-mai' (A and C) and red-purple-flowered mutant No. 7 (B and D). Arrows indicate the basal 'eye' with deep coloring, and arrowheads indicate the lighter colored 'slip' of the petals. Scale bars are 40 mm.

Table 1. Effects of carbon ion beam irradiation on regeneration rate and mutation induction in *in vitro* cultures of etiolated petiole of fragrant cyclamen (*Cyclamen persicum*×*C. purpurascens*) 'Kaori-no-mai'.

Irradiation dose (Gy)	Number of etiolated petioles irradiated	Number of plantlets generated (%)	Number of M_1 plants grown to bloom	Number of M_2 seeds obtained (Number of M_2 seeds sown for examination)	Number of M_2 plants grown to bloom	Number of M_2 plants producing red-purple flowers
0	294	270 (92)	58	767 (200)	52	0
0.5	396	372 (94)	53	740 (200)	61	0
1	526	452 (86)	75	892 (200)	90	0
2	240	196 (82)	30	537 (200)	68	9
4	81	54 (67)	3	0	—	—
8	74	39 (53)	1	0	—	—
16	36	7 (19)	2	0	—	—

transferred to 90-mm plastic Petri dishes containing fresh AS medium to induce adventitious shoots and cultured at 25°C in the dark. Adventitious shoots generated from the etiolated petioles were transferred to AR medium to induce adventitious roots and cultured at 25°C under fluorescent illumination (34 μmol m^{-2} S^{-1}) with a 16-h light period and 8-h dark period until M_1 plantlets generated.

The M_1 plantlets were transplanted into 60-mm plastic pots containing vermiculite and were kept in plastic containers. They were then covered with a plastic film and were acclimatized for 4 weeks under the same conditions used to induce adventitious roots. The M_1 plantlets were then transferred to a greenhouse under natural daylight and were transplanted to 105-mm plastic pots containing steam-sterilized mixed soil (fertilized red soil : leaf mould = 40 : 60). They were then grown to maturity in the greenhouse. M_2 seeds were obtained by self-pollination of each M_1 plant. The M_2 seeds were surface-sterilized with 1% sodium hypochlorite and sown in 200-cell trays containing Metro Mix 350 growing medium (Sun Glo Horticulture Distribution Inc., McCormick, South Carolina, USA) and were maintained in a greenhouse at 20–25°C in the dark. The M_2 seedlings were transplanted to 90-mm plastic pots containing the same 40 : 60 soil mix described above and were grown in a greenhouse under natural daylight. Thereafter, the M_2 seedlings were transplanted to 120-mm plastic pots and grown to maturity in the greenhouse.

The effects of the carbon-ion beam irradiation dosage on regeneration rate and induction of mutations in the M_1 and M_2 plants are presented in Table 1. The purple petal coloration and other properties in all the M_1 plants resembled those of Kaori-no-mai. Among the M_2 population, 68 individuals from the 2-Gy irradiation dosage were brought to bloom, and of these, 9 plants displayed different petal coloration from Kaori-no-mai but the same flower shape, flower size, leaf color, and fragrance as Kaori-no-mai (Figure 1A, B). The flowers of the 9 plants were red-purple and one plant (mutant No. 7) was used for anthocyanin analysis.

Anthocyanins of Kaori-no-mai and mutant No. 7 were analyzed by means of HPLC, TLC, and ESIMS. Petals of these plants were separated into slips and eyes (Figure 1C, D). Each tissue was extracted with 10% acetic acid. The extract was analyzed on an HP1100 system with a photodiode array detector (Agilent Technologies, CA, USA) and a COSMOSIL ODS column (4.6 mm×250 mm, Nacalai Tesque, Kyoto, Japan) at 40°C at a flow rate of 0.8 mL/min. Absorption spectra were monitored at 200–600 nm. A linear gradient of 25%–45% solvent B (1.5% H_3PO_4, 20% CH_3CO_2H, 25% CH_3CN) in solvent A (1.5% H_3PO_4) was run for 50 min. Anthocyanins were quantified based on the absorption at 530 nm, and their concentrations were calculated as cyanidin 3-rutinoside equivalent. The extracts of mutant No. 7 were analyzed by cellulose TLC (Merck, Darmstadt, Germany) with two solvents, *n*-butanol : acetic acid : water (4 : 1 : 2) and 10% acetic acid.

Each different one major the anthocyanins was detected in the slips of Kaori-no-mai and mutant No. 7 (Figure 2). Because the retention time, UV-Vis spectra, and R_f values of the anthocyanins coincided with those of standard samples, the major anthocyanin of 'Kaori-no-mai' was identified as malvidin 3,5-diglucoside, whereas that of mutant No. 7 was identified as delphinidin 3,5-diglucoside (Table 2, Figure 2). The extract of mutant No. 7 was passed through an ODS Sep-Pak cartridge, and the purified anthocyanin was analyzed by ESIMS (Thermo Quest, CA, USA). The ESIMS datum (*m*/*z* 627) confirmed the identification of delphinidin 3,5-diglucoside. The difference in the anthocyanin components in slips clearly indicates that mutant No. 7 loses methylation activity at the anthocyanin 3′ and 5′-hydroxyl groups (Table 2). Slips of mutant No. 7 retained similar coloration depth as those of Kaori-no-mai and there was little significant difference in anthocyanin

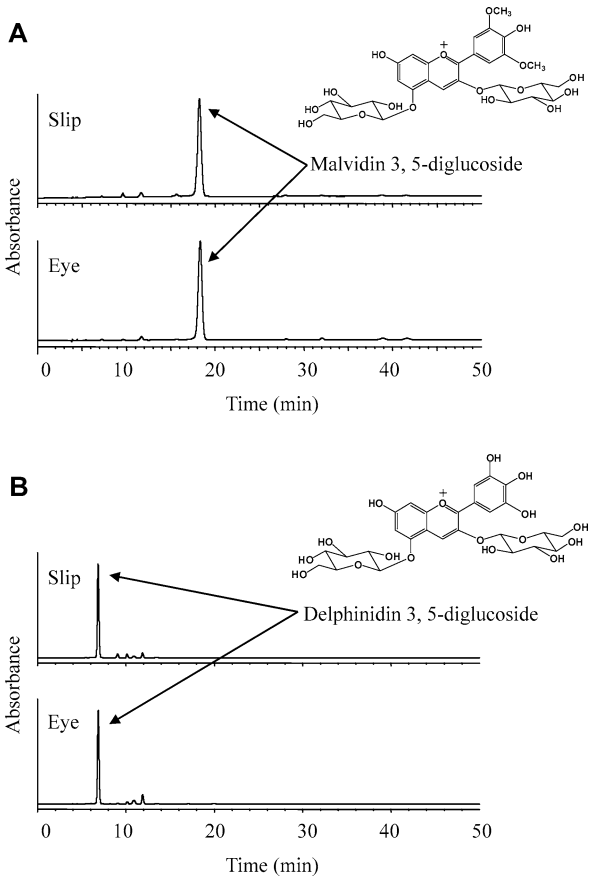

Figure 2. HPLC elution profiles detected by absorbance at 530 nm and structures of anthocyanins in the slips and eyes of (A) 'Kaori-no-mai' and (B) mutant No. 7. As the major anthocyanins, malvidin 3,5-diglucoside and delphinidin 3,5-diglucoside were detected in 'Kaori-no-mai' and mutant No. 7, respectively.

Table 2. Chromatographic and spectral properties of cyclamen anthocyanins.

Plant tissue	Anthocyanin	Concentration (μmol g^{-1} fresh weight)	Rt[a] (min)	R_f values		λmax (nm)	
				BAW[b]	10% AcOH	UV	Vis
'Kaori-no-mai'							
Slip	Malvidin 3,5-diglucoside	3.2 ± 0.3	17.0	0.31	0.40	275	525
Eye	Malvidin 3,5-diglucoside	4.8 ± 0.2	17.0	0.31	0.40	275	525
Mutant No. 7							
Slip	Delphinidin 3,5-diglucoside	4.3 ± 0.4	6.8	0.10	0.23	274	522
Eye	Delphinidin 3,5-diglucoside	6.8 ± 0.6	6.9	0.10	0.23	274	522
Standard samples							
	Malvidin 3,5-diglucoside		17.0	0.31	0.40	275	525
	Delphinidin 3,5-diglucoside		6.9	0.10	0.23	274	522

[a] Retention time
[b] BAW, *n*-butanol : acetic acid : water, 4 : 1 : 2

concentrations between Kaori-no-mai and mutant No. 7 (Table 2), indicating that the mutation caused by ion-beam irradiation has little influence on anthocyanin concentration.

The petals of many horticulturally important cyclamen cultivars show purple and red coloration. Seven anthocyanins have been identified as the major pigments of cyclamen petals: malvidin 3-glucoside, malvidin 3,5-diglucoside, peonidin 3-glucoside, peonidin 3-neohesperidoside, peonidin 3,5-diglucoside, cyanidin 3-glucoside, and cyanidin 3,5-diglucoside (Sugimura et al. 1997; Webby and Boase 1999). These anthocyanin components yield corresponding petal colorations: malvidin 3,5-diglucoside yields purple, malvidin 3-glucoside yields red-purple, and cyanidin- and peonidin-type anthocyanins yield red (Sugimura et al. 1997). The purple petals of Kaori-no-mai also contain malvidin 3,5-diglucoside. The red-purple color of mutant No. 7 caused by delphinidin 3,5-diglucoside is similar to that of the cultivars Wine and Wine-no-kaori, which possess malvidin 3-glucoside as their major pigment (authors' unpublished data). However, the red-purple coloration of mutant No. 7 is different from those of these existing cyclamen cultivars.

Eyes are distinguished from slips by their deeper coloration at the base of petals (Figure 1C, D). The major anthocyanin in eye was the same as slip in each plant (Table 2). Consistent with the deeper coloration, the eyes contained higher concentrations of anthocyanin than the slips in both plants. As in the slips, anthocyanin concentration in eyes differed only slightly between Kaori-no-mai and mutant No. 7. Therefore, anthocyanin production is probably underlain by the same biosynthetic regulation in both slips and eyes.

We produced a fragrant cyclamen in which the major anthocyanin component was changed from malvidin 3,5-diglucoside to delphinidin 3,5-diglucoside by carbon-ion-beam irradiation. Hugueney et al. (2009) reported that in grapevine, methylation of the 3′-and 5′-hydroxyl groups of anthocyanins was catalyzed by *O*-methyltransferase. In the next phase of our research we will examine the genomic connection between *O*-methyltransferase and the structure of the 3′-and 5′-hydroxyl groups of the anthocyanins in mutant No. 7. We will also examine the components of the scent compounds in more detail. We expect that mutant No. 7 will not only become a new commercial cultivar with an unprecedented combination of attributes, but it could also provide a valuable genetic resource for breeding a new red-purple coloration cultivars because generation of cyclamen containing mostly the delphinidin type anthocyanin is a breakthrough in cyclamen breeding. This study represents a successful example of mutation breeding with the ion-beam technique.

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