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Morphological variation in *Tricyrtis hirta* plants regenerated from heavy ion beam-irradiated embryogenic calluses

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Abstract In order to induce horticulturally valuable mutants in the Liliaceous ornamental *Tricyrtis hirta*, embryogenic calluses of this species were irradiated with $^{12}C^{+6}$ ion beams. Morphological characterization was performed on 35, 37 and 15 plants regenerated from calluses irradiated with 5, 10 and 20 Gy ion beams, respectively, and on 10 plants from non-irradiated calluses after 3 years of cultivation in pots. No plants were regenerated from calluses irradiated with 50 Gy ion beams. There were no large differences in the mean values of leaf length, leaf width, soil and plant analyzer development (SPAD) value of leaves, flower length and flower diameter between the control (division-derived plants from the mother plant of the embryogenic calluses) and the irradiation treatments at different doses. On the other hand, the mean number of shoots per plant increased, and the mean shoot length and the mean number of nodes per shoot decreased in the irradiation treatments. The mean number of flowers per plant was increased in the 20 Gy irradiation treatment. For most morphological characteristics investigated, the variation spectrum widened with increase in the irradiation dose. Several horticulturally attractive variations such as dwarfism, slender and deep green leaves, and large flowers were observed in regenerants from the irradiation treatments, and these variations were stable after additional 2 years of cultivation in pots or garden. Thus mutation induction by heavy ion beam irradiation of embryogenic calluses is a valuable tool for improving horticultural value of *T. hirta*.

Key words: Horticultural characterization, $^{12}C^{+6}$ ion beam irradiation, Japanese toad lily, mutation breeding, somatic embryogenesis.

Tricyrtis hirta, sometimes called 'Japanese toad lily', is a liliaceous perennial plant native to Japan. This plant has erect to arching shoots and beautiful foliage, and produces exotic starry flowers in autumn. Because of these characteristics, *T. hirta* has recently become popular as an ornamental for pot and garden uses. However, no systematic breeding has yet been conducted in *T. hirta*, and there are only a few variations in horticultural traits such as plant form, flower color and flower shape among cultivated *T. hirta* strains. Recently, highly embryogenic callus cultures of *T. hirta* were established (Nakano et al. 2004), and somaclonal variation in plants regenerated from 1-year-old embryogenic callus cultures was demonstrated (Nakano et al. 2006b). In addition, an efficient *Agrobacterium*-mediated transformation system of *T. hirta* was developed using the embryogenic callus cultures (Adachi et al. 2005; Nakano et al. 2006a; Mori et al. 2008).

Induction and selection of mutants is a powerful tool for plant breeding as well as for physiological and molecular studies. For mutation induction, X-ray irradiation, gamma ray irradiation and chemical treatments have been carried out in a wide range of plants (van Harten 1998, 2002). Heavy ion beams have recently been used as a new mutagen in plants because of their very high LET and RBE compared with gamma rays. Heavy ion beam-induced new cultivars have already been developed in several ornamentals such as

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; FCM, flow cytometry; FW, fresh weight; LET, high linear energy transfer; MS, Murashige and Skoog; PGR, plant growth regulator; RBE, relative biological effectiveness; SPAD, soil and plant analyzer development.

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Dahlia pinnata (Hamatani et al. 2001), *Verbena hybrida* (Suzuki et al. 2002), *Petunia hybrida* (Miyazaki et al. 2002), *Dianthus caryophyllus* (Okamura et al. 2003), *Torenia* hybrid (Miyazaki et al. 2006) and *Dianthus chinensis* (Sugiyama et al. 2008). We previously examined the effect of $^{12}C^{+6}$ ion beam irradiation on the growth and development of embryogenic calluses of *T. hirta* (Nakano et al. 2010). In the present study, horticultural characterization of plants regenerated from the ion beam-irradiated embryogenic calluses was carried out for evaluating the validity of heavy ion beam irradiation in the breeding program of *T. hirta*.

Materials and methods

Tepal-derived, highly embryogenic calluses of a diploid strain of *T. hirta* (Nakano et al. 2004) were maintained for 8 months by monthly subculturing to half-strength MS medium (Murashige and Skoog 1962) containing 4.5 μM 2,4-D at 25°C in the dark. For irradiation, 0.3 g FW of calluses were placed on fresh medium of the same composition in a plastic Petri dish (6 cm in diameter). They were irradiated with 5, 10, 20 or 50 Gy of $^{12}C^{6+}$ ions (135 MeV nucleon^{-1}; LET, 23 keV μm^{-1}) at the RIKEN RI-beam Factory (RIBF) (Nakano et al. 2010). Two days after irradiation, calluses were transferred to half-strength MS medium lacking PGRs to induce plant regeneration. Non-irradiated embryogenic calluses were also transferred to PGR-free, half-strength MS medium. Regenerated plantlets were acclimatized, separately transplanted to pots (18 cm in diameter), and cultivated in the greenhouse as previously described (Nakano et al. 2006b). Divisions, each containing one small shoot, from the mother plant of the embryogenic calluses were transplanted to pots and cultivated as controls under the same conditions.

Three years after cultivation in the greenhouse, morphological characterization and ploidy level analysis were performed on regenerants from irradiated and non-irradiated embryogenic calluses and the control, division-derived plants according to Nakano et al. (2006b). For morphological characterization, number of shoots per plant, mean shoot length of the longest 3 shoots, mean number of nodes per shoot of the longest 3 shoots, mean leaf length and width of randomly selected 9 leaves (3 leaves each from the longest 3 shoots), number of flowers per plant, mean flower length and diameter of randomly selected 3 flowers were recorded for each plant at the flowering stage. Mean SPAD value of randomly selected 3 leaves was also measured for each plant using a chlorophyll meter (SPAD-502; Fujiwara Scientific Co., Tokyo, Japan). This value has a high correlation with chlorophyll concentration on a leaf area basis, and is commonly used to predict chlorophyll concentration in various plant species (Campbell et al. 1990; Koike et al. 2003). Ploidy level of each plant was determined by FCM analysis of leaf tissues (Saito et al. 2003) using a flow cytometer PA (Partec, GmbH-Münster, Germany).

Following morphological characterization, some regenerants were divided into 2 pieces, each containing 5–8 shoots. One was transplanted to a new pot (18 cm in diameter) and cultivated in the greenhouse, and the other was transplanted to the garden under deciduous trees in the Faculty of Agriculture, Niigata University.

Results and discussion

As reported previously (Nakano et al. 2010), more than 80% of somatic embryos produced from non-irradiated calluses and from calluses irradiated with 5 and 10 Gy of $^{112}C^{+6}$ ions subsequently developed into plantlets. On the other hand, only about 40% of somatic embryos derived from calluses irradiated with 20 Gy ion beams developed into plantlets. No somatic embryos were produced from calluses irradiated with 50 Gy ion beams. Forty, 40 and 20 plantlets regenerated from calluses irradiated with 5, 10 and 20 Gy ion beams, respectively, and 10 plantlets from non-irradiated calluses were transplanted to pots and cultivated in the greenhouse in September. During 3 years of cultivation, 2, 2 and 5 regenerants from the 5, 10 and 20 Gy treatments, respectively, showed poor growth and ultimately died.

Three years after cultivation, ploidy level of regenerants was initially analyzed. All the 10 regenerants from non-irradiated calluses were diploid ($2n=2x=26$) as the control, division-derived plants. Among 38 regenerants from the 5 Gy treatment, 3 were tetraploid ($2n=4x=52$) and the other 35 were diploid. Among 38 regenerants from the 10 Gy treatment, 1 was tetraploid and the other 37 kept the diploid level. No tetraploids were detected among 15 regenerants from the 20 Gy treatment. Chromosome observation of randomly selected regenerants confirmed the results of FCM analysis (data not shown). All the 4 tetraploid regenerants had longer shoots, thicker stems, larger and deeper green leaves, and larger flowers compared with the control, division-derived plants. Tetraploid plants have already been regenerated from long-term (1-year-old) embryogenic callus cultures of *T. hirta*, and they showed similar morphological characteristics to those of the tetraploid regenerants obtained in the present study (Nakano et al. 2006b). Since relatively long-term (8-month-old) embryogenic callus cultures were used as a target material for irradiation in the present study, the 4 tetraploid regenerants may be resulted from somaclonal variation rather than from ion beam irradiation. Therefore, detailed morphological characterization was not performed on the 4 tetraploid regenerants.

Thirty-five, 37 and 15 diploid regenerants from calluses irradiated with 5, 10 and 20 Gy ion beams, respectively, and 10 diploid regenerants from non-irradiated calluses were then subjected to morphological characterization. Although most of these regenerants produced flowers after 3 years of cultivation, 1 regenerant from the 20 Gy treatment produced no flowers even after 5 years of cultivation. All the regenerants from irradiated and non-irradiated embryogenic calluses had erect shoots as the control, division-derived plants. Table 1 shows the mean values of several morphological

Table 1. Morphological characteristics of *Tricyrtis hirta* plants regenerated from $^{12}C^{+6}$ ion beam-irradiated embryogenic calluses after 3 years of cultivation in pots[a].

Irradiation dose (Gy)	No. of plants examined	No. of shoots per plant	Shoot length (cm)[b]	No. of nodes per shoot[b]	Leaf length (cm)[c]	Leaf width (cm)[c]	Leaf SPAD value[d]	No. of flowers per plant	Flower length (cm)[e]	Flower diameter (cm)[e]
Control[f]	5	10.0 (8–12)	35.8 (32.3–36.5)	15.0 (14.3–16.6)	6.8 (6.3–6.9)	2.2 (1.9–2.5)	30.2 (27.4–34.1)	14.0 (11–19)	2.6 (2.5–2.7)	3.6 (3.3–3.8)
0[g]	10	10.2 (7–13)	36.0 (33.9–38.7)	15.1 (13.7–16.3)	6.8 (6.0–7.3)	2.2 (1.8–2.5)	30.6 (26.3–35.5)	13.5 (10–18)	2.6 (2.4–2.8)	3.6 (3.3–3.8)
5	35	11.7 (4–26)	34.0 (27.8–43.6)	13.8 (10.3–16.3)	6.9 (6.3–7.4)	2.3 (2.0–2.8)	30.2 (26.0–37.3)	13.4 (3–27)	2.6 (2.5–2.9)	3.7 (3.4–4.0)
10	37	14.9 (5–49)	32.6 (19.3–38.8)	13.1 (9.0–16.3)	6.7 (5.1–7.9)	2.2 (1.9–2.5)	30.7 (25.3–38.7)	13.4 (1–28)	2.6 (2.1–2.9)	3.6 (3.3–4.5)
20	15	13.4 (5–22)	26.4 (16.9–39.7)	13.7 (11.3–16.3)	6.7 (6.0–7.6)	2.2 (1.7–2.7)	30.9 (28.2–35.3)	18.6 (0–49)	2.6 (2.5–2.7)	3.4 (2.7–3.8)

[a] Only diploid plants were examined. Values, except for those of flower length and flower diameter for the 20 Gy treatment, represent the mean of 5, 10, 35, 37 and 15 plants for the control, non-irradiation, 5 Gy, 10 Gy and 20 Gy treatments, respectively. Values of flower length and flower diameter for the 20 Gy treatment represent the mean of 14 plants, because 1 plant of this treatment produced no flowers. Parenthesized values represent the minimum and the maximum.

[b] The longest 3 shoots were investigated for each plant.

[c] Randomly selected 9 leaves were investigated for each plant.

[d] Randomly selected 3 leaves were investigated for each plant.

[e] Randomly selected 3 flowers were investigated for each plant.

[f] Divisions from the mother plant of embryogenic calluses.

[g] Plants regenerated from non-irradiated embryogenic calluses.

characteristics within each irradiation treatment. For all the characteristics examined, no differences were observed between the control, division-derived plants and regenerants from non-irradiated calluses. No large differences in the mean values of leaf length (control, 6.8 cm; irradiation treatments, 6.7–6.9 cm), leaf width (control, 2.2 cm; irradiation treatments, 2.2–2.3 cm), SPAD value of leaves (control, 30.2; irradiation treatments, 30.2–30.9), flower length (control, 2.6 cm; irradiation treatments, 2.6 cm) and flower diameter (control, 3.6 cm; irradiation treatments, 3.4–3.7 cm) were observed between the control (division-derived plants) and the irradiation treatments at different doses. On the other hand, the mean number of shoots per plant (control, 10.0; irradiation treatments, 11.7–14.9) increased, and the mean shoot length (control, 35.8 cm; irradiation treatments, 26.4–34.0 cm) and the mean number of nodes per shoot (control, 15.0; irradiation treatments, 13.1–13.8) decreased in the irradiation treatments. Although no differences in the mean number of flowers per plant were observed between the control (14.0) and the irradiation treatments at 5 or 10 Gy (13.4), the mean number increased in the 20 Gy irradiation treatment (18.6). For the number of shoots per plant, shoot length, number of nodes per shoot, leaf SPAD value and number of flowers per plant, the variation spectrum widened with increase in the irradiation dose.

Table 2 shows visibly apparent variations, compared with the control, division-derived plants, in regenerants from ion beam-irradiated embryogenic calluses, and the number of regenerants showing each type of variation. No apparent variations were observed in plants regenerated from non-irradiated embryogenic calluses. Among irradiation treatments at different doses, regenerants with visibly apparent variations were most frequently obtained in the 10 Gy treatment (32.4%). In addition, most various types of variation were observed in the 10 Gy treatment. Since no apparent decrease in the efficiencies of somatic embryo production from irradiated calluses and conversion of somatic embryos into plantlets were observed in the 10 Gy $^{12}C^{+6}$ irradiation treatment (Nakano et al. 2010), this treatment may be appropriate for efficient induction of variations in embryogenic calluses of *T. hirta*.

Among the variation types, increased numbers of shoots per plant, dwarfism (Figure 1), decreased numbers of nodes per shoot, increased or decreased numbers of flowers per plant were often obtained (Table 2). Less frequently, small, slender and deep green leaves (Figure 2), small round leaves (Figure 2), large or small flowers (Figure 3), pale flower color were obtained. Several plants simultaneously showed different types of variation: for example, 1 regenerant from the 10 Gy treatment (line C10-9) showed a dwarf plant form with increased numbers of shoots and flowers per plant, and

Table 2. Visibly apparent variations observed in *Tricyrtis hirta* plants regenerated from $^{12}C^{+6}$ ion beam-irradiated embryogenic calluses and the number of regenerants showing each type of variation after 3 years of cultivation in pots[a].

	Irradiation dose (Gy)			
	0[b]	5	10	20
No. of regenerants examined	10	35	37	15
No. of regenerants with apparent variations (%)	0 (0)	5 (14.3)	12 (32.4)	4 (26.7)
Variation type				
Increased numbers of shoots per plant	0	2	6	1
Dwarfism	0	2	8	1
Decreased numbers of nodes per shoot	0	1	3	0
Small, slender and deep green leaves	0	0	1	0
Small and round leaves	0	0	1	0
No flowering	0	0	0	1
Increased numbers of flowers per plant	0	1	3	4
Decreased numbers of flowers per plant	0	1	2	1
Large flowers	0	0	1	0
Small flowers	0	0	1	1
Pale flower color	0	1	1	0

[a] Only diploid regenerants were examined.
[b] Plants regenerated from non-irradiated embryogenic calluses.

Figure 1. Variations in the plant height of *Tricyrtis hirta* plants regenerated from $^{12}C^{+6}$ ion beam-irradiated embryogenic calluses after 3 years of cultivation in pots. Left, a regenerant without variations from non-irradiated calluses; middle, a dwarf regenerant from the 10 Gy irradiation treatment (line C10-9); right, a dwarf regenerant from the 10 Gy irradiation treatment (line C10-31). Bar=15 cm.

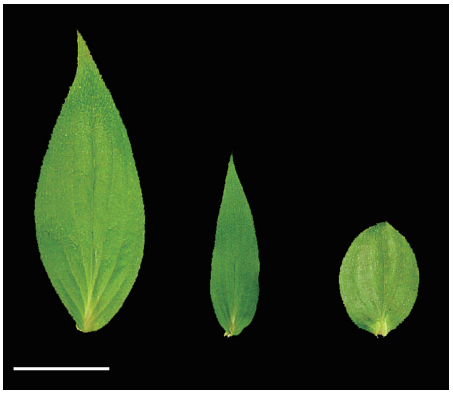

Figure 2. Variations in the leaf morphology of *Tricyrtis hirta* plants regenerated from $^{12}C^{+6}$ ion beam-irradiated embryogenic calluses after 3 years of cultivation in pots. Left, a leaf without variations of a regenerant from non-irradiated calluses; middle, a small, slender and deep green leaf of a regenerant from the 10 Gy irradiation treatment (line C10-9); right, a small and round leaf of a regenerant from the 10 Gy irradiation treatment (line C10-31). Bar=2 cm.

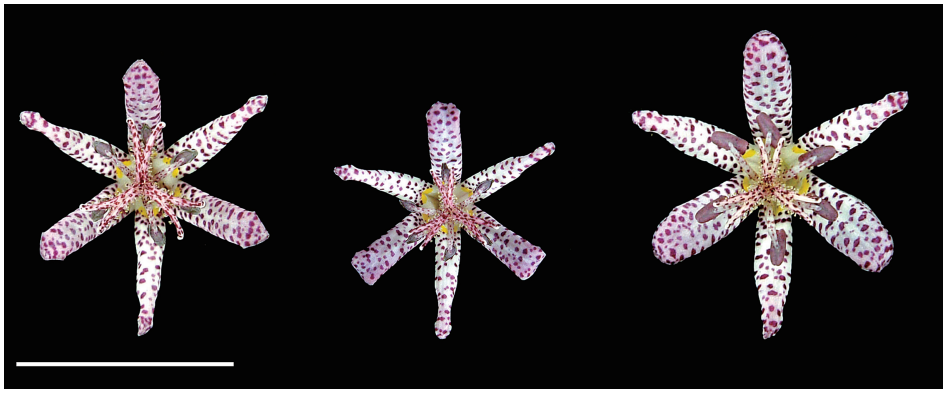

Figure 3. Variations in the flower size of *Tricyrtis hirta* plants regenerated from $^{12}C^{+6}$ ion beam-irradiated embryogenic calluses after 3 years of cultivation in pots. Left, a flower without variations of a regenerant from non-irradiated calluses; middle, a small flower of a regenerant from the 20 Gy irradiation treatment (line C20-8); right, a large flower of a regenerant from the irradiation treatment (line C10-12). Bar=2 cm.

Table 3. Several *Tricyrtis hirta* plants with apparent variations regenerated from $^{12}C^{+6}$ ion beam-irradiated embryogenic calluses[a]

Line	Irradiation dose (Gy)	Observed variations
C5-32	5	Pale flower color
C10-9	10	Dwarfism; increased number of shoots per plant; small, slender and deep green leaves; increased number of flowers per plant
C10-12	10	Large flowers
C10-15	10	Pale flower color
C10-31	10	Dwarfism; increased number of shoots per plant; small and round leaves; increased number of flowers per plant
C10-33	10	Dwarfism
C20-8	20	Dwarfism; small flowers

[a] Only diploid regenerants were examined.

had small, slender and deep green leaves (Table 3). After 3 years of cultivation in pots, 7 regenerants with visibly apparent variations (Table 3) were selected, divided and transplanted to pots and garden. Divisions of all the 7 lines grew well and stably maintained the variations following additional 2 years of cultivation both in pots and garden (data not shown).

In our previous report, 2 types of somaclonal variants were observed among regenerants from 1-year-old embryogenic calluses of *T. hirta*: one type was a tetraploid variant, which was also obtained in the present study as described above; and the other type was a diploid, severely dwarf variant with crimped leaves and many malformed flowers (Nakano et al. 2006b). Although several dwarf variants were regenerated from ion beam-irradiated embryogenic calluses in the present study, all of them had neither crimped leaves nor malformed flowers, and were clearly distinguishable from the severely dwarf somaclonal variant reported previously (Nakano et al. 2006b). In addition, no severely dwarf variants with crimped leaves and malformed flowers were regenerated from non-irradiated embryogenic calluses in the present study. Thus the dwarf variants obtained in the present study may be resulted from ion beam irradiation rather than from somaclonal variation. To date, various mutation phenotypes have been reported in mutational studies using various plant materials and mutagens, among which dwarfism and chlorophyll-related mutations such as albinism are typical mutation phenotypes often obtained (Gottschalk and Wolff 1983; van Harten 2002). Dwarfism is also observed most frequently in the present study, although no chlorophyll-related mutations were obtained. In our preliminary experiments, exogenous application of gibberellic acid to several dwarf variants (C10-9, C10-31 and C20-8) resulted in shoot elongation to some extents (data not shown), indicating that the endogenous gibberellin level might decrease in these dwarf variants.

In mutation breeding, chimerism is one of the major problems (Godo et al. 2007; van Harten 2002), and repeated cutting back of chimerical shoots (Yamaguchi et al. 2003) or adventitious shoot induction from chimerical organs (Miyazaki et al. 2002) is often required for establishing solid mutants. However, in the present study, no possible chimerical plants were obtained, probably due to utilization of embryogenic calluses as a target material for ion beam irradiation. It has generally been accepted that somatic embryos have an ultimate single cell origin (Nomura and Komamine 1985). Therefore, embryogenic calluses may be an appropriate material for mutation induction avoiding problems of chimerism, although additional time and effort are required for their induction and maintenance.

In the present study, several horticulturally attractive variations such as dwarfism, small, slender and deep green leaves, and large flowers were observed in plants regenerated from ion beam-irradiated embryogenic calluses of *T. hirta* after 3 years of cultivation in pots. In addition, such attractive variations were stably maintained after additional 2 years of cultivation in pots or garden. Therefore, mutation induction by heavy ion beam irradiation of embryogenic calluses is an effective tool for improving horticultural values of *T. hirta*. Further characterization and vegetative propagation of several promising variants obtained in the present study are now in progress.

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