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Original Paper

Heavy-ion beam-induced sterile mutants of verbena (*Verbena*×*hybrida*) with an improved flowering habit

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Abstract Sterile mutants of *Verbena*×*hybrida* were isolated at high frequency from nodal cultures of developed plants irradiated with heavy-ion beams. Sixty four *in vitro*-cultured nodes of fertile cultivars 'Temari Sakura' (FS), 'Temari Coral Pink' (FC) and 'Temari White' (FW) were irradiated with 1 to 10Gy of ^{14}N-ion beam (1890MeV). Lateral shoot development of FS, FC and FW was not affected by irradiation with up to 10Gy. After open-pollination, shoots with inflorescence forming unenlarged ovaries were selected and propagated several times by cutting. Shoots were grown to flowering and the selection process for isolating stable sterile mutants was carried out by the same method. Finally, one mutant out of 104 FS lateral shoots (5Gy), one mutant out of 115 FC shoots (5Gy) and 3 mutants out of 108 FC shoots (10Gy) were successfully isolated. With the exception of sterility all these mutants showed normal morphology. Two sterile mutants SS and SC, which were isolated from 5Gy-irradiated FS and FC, respectively, were characterized by their flowering habits. These two mutants grew well, had a larger number of inflorescences, and a better longevity compared with their parental cultivars. These results show that heavy-ion beam irradiation is an excellent tool for isolating sterile mutants without alterations in others important traits at a high frequency. In addition, the characterization of SS and SC indicated that they have different sterile phenotypes: male and female gametes of SS are non-functional, in contrast, SC exhibits self-incompatibility, which results in mutants unable to produce seeds.

Key words: Heavy-ion beam, self-incompatibility, *Verbena*×*hybrida*.

Verbena, a genus of the Verbenaceae family, consists of approximately 250 species worldwide. Most of these species are native to temperate, tropical or subtropical regions of South, Central and North America. Numerous horticultural cultivars of verbenas (i.e. *Verbena*×*hybrida*) have been bred by artificial crossing among several species and were popular for hanging basket plants and garden uses because of their wealth of flower colors and broad range of growth habits. During the last decade, Suntory Flowers Ltd. (Tokyo, Japan) has developed new cultivars of the verbena Temari® series that have characteristics such as resistance to powdery mildew and heat tolerance that are superior to those of common garden verbena cultivars. Besides, most of the Temari® cultivars have a continuous flowering habit that is characterized by a large number of inflorescences from spring until autumn in the temperate regions. However, some cultivars sometimes show a decrease in the number of inflorescences. It was observed that they produce self-seeds at a high frequency, and the number of inflorescences tends to decrease after seed setting. Usually, in monocarpic plants, the production of fruits and seeds causes plant senescence (Noodén 1980), and it has been reported in soybean that the removal of fertilized flowers or young pods leads to an increased number of flowers compared with untreated plants (Noodén 1984). Although perennial verbenas are not monocarpic plants, we believe that the number of inflorescences in verbena cultivars might be also increased by the repression of seed setting.

The induction of sterile mutants seems to be one of the most effective methods to improve the flowering habits of self-compatible verbenas. Radiations such as gamma rays and X-rays have been widely used for mutation induction in numerous plant species (van Harten 1998). Because of their lower biological effects compared with low linear energy transfer (LET) radiations such as gamma rays, X-rays and electrons (Kraft et al. 1992;

Abbreviations: LET, linear energy transfer; MS, Murashige and Skoog.
This article can be found at http://www.jspcmb.jp/

Tanaka et al. 1997), high LET heavy-ion beams are being frequently used for improving ornamental plants (Hamatani et al. 2001; Hara et al. 2003; Miyazaki et al. 2006) and for genetic analysis in crop science (Honda et al. 2006; Shitsukawa et al. 2007). We report here the high frequency isolation of sterile verbena mutants at by heavy-ion beam irradiation, and the characterization of their flowering habits and sterile phenotypes.

Materials and methods

Plant materials and mutation induction

Fertile verbena cultivars *Verbena*×*hybrida* 'Temari Sakura' (FS), 'Temari Coral Pink' (FC) and 'Temari White' (FW) (Suntory Flowers Ltd., Tokyo, Japan) were used. *In vitro* plantlets of these cultivars were maintained on MS (Murashige and Skoog 1962) medium containing 3% sucrose and 0.8% agar, and their nodes, which had two lateral meristems at the base of two opposite leaves, were subjected to irradiation treatment. Sixty-four single nodes cultured in a plastic dish were irradiated with 0, 1, 2, 5 or 10 gray (Gy) of nitrogen ion ($^{14}N^{7+}$) beams at 1890 MeV accelerated by RIKEN Ring Cyclotron at RI-Beam Factory (RIKEN Nishina Center, Wako, Japan). The LET of N ion beams corresponded to 31 keVμm^{-1}.

Isolation of sterile mutants

After the irradiation treatment, the cultures were incubated at 25°C under a 16/8-h (light/dark) photoperiod with light provided by fluorescent lamps at an intensity of 60 μmolm^{-2} s^{-1}. Twenty days after the irradiation, the frequency of shoot development from single nodal culture was determined. All shoots developed from lateral meristems after the irradiations were cut into a single shoot, and they were rooted, acclimatized, transplanted to 128-cell trays containing growing media (peat: perlite: vermiculite=60 : 20 : 20), and grown to flowering in the greenhouse. After the open-pollination, shoots with seed-producing inflorescences were immediately removed, and shoots with inflorescences that formed unenlarged ovaries were selected and then propagated several times by cutting. These cuttings were grown to flowering and the next selection process to isolate mutant lines with stable sterility was carried out by the same methods.

Characterization of flowering habits

Mutant lines with stable sterility were propagated vegetatively by cutting and compared with their parental cultivars. Shoots consisting of four nodes and an apical bud were cut from mature plants and incubated for rooting in distilled water. After one month, three rooted cuttings were transplanted into one plastic pot (15×20 cm) containing growing media, and cultured in the greenhouse. They were cultured at 25/22°C under a 16/8-h (light/dark) photoperiod with light provided by the sun and fluorescent lamps at an intensity of at least 60 μmolm^{-2} s^{-1}. Sterile mutants and parental cultivars were evaluated for Flower shape and color, duration of inflorescence life (from the time that the first flower opened to the time that the last flower wilted), the number of floret per inflorescence and the number of inflorescences in every three plants growing in one pot during the blooming season. Additionally, three rooted cuttings of mutant lines and parental cultivars propagated in the same way were transplanted into plastic pot (24×17.5 cm) containing growing media. They were grown in the open air from the beginning of April to late October and their flowering habits and resistance to climate and diseases evaluated.

Characterization of the sterile phenotype

Fertile parental cultivars and sterile mutants were self- and cross-pollinated in order to investigate their pollen and embryo sac fertility. In addition, fertile cultivars of Temari® series (FW) and 'Temari Lilac' (FL) were used for cross-pollination tests. Plants were cultured at 25/22°C under a 16/8-h (day/night) photoperiod with light provided by fluorescent lamps at an intensity of 60 μmolm^{-2} s^{-1} in a growth chamber.

Mature pollen grains harvested at anthesis were stained with 1% aceto-carmine and were observed under a light microscope for the assessment of pollen viability. Open florets and very immature florets were removed from inflorescences before pollination. As for the maternal lines, sepals, colloras and anthers were removed from immature florets that appeared to be, based on size, color and position in the inflorescence, within one day of opening. Whole inflorescences with emasculated florets were washed with distilled water and covered with paper bags for one day. Controlled pollinations of selfing and crossing were carried out with fresh pollen grains harvested at anthesis, and then inflorescences were covered again. In non-controlled pollinations, whole inflorescences with immature florets were covered with paper bags to avoid cross-pollination. Seed set frequency was analyzed one month after pollination.

Pollen germination and pollen tube growth were evaluated in order to analyze the pollen-pistil interaction. Pistils containing ovaries were collected some hours after selfing and fixed in FAA (70% ethanol: glacial acetic acid: formaldehyde=90 : 5 : 5, v/v/v) for more than 24 h. After rinsing with distilled water, they were cleared in 1N KOH at room temperature for one day. Then they were rinsed twice in water and stained for 2 h with 0.1% aniline blue solution prepared in 0.1 M K_3PO_4 (Singh 2003). Samples were gently squashed in a drop of 50% glycerine under a cover glass and observed under a UV light fluorescence microscope (OLYMPUS IX70).

Results and discussion

Isolation of sterile mutants

Effects of the irradiation treatment on the development of lateral shoots and the isolation of sterile mutant lines from FS, FC, and FW are shown in Table 1. In the control nodes of cultivars without irradiations, about 80% of all lateral meristems developed shoots within 20 days. The percentage of shoot development decreased to 27.3% and 0% by the 10 Gy irradiation treatments of FS and FW, respectively. In contrast, FC was not affected by any of the radiation dose tested. Suzuki et al. (2004 and 2005) and van Harten (1998) reported that radiosensitivity, measured as survival rate and mutation frequency was different among cultivars.

All shoots developed from the lateral meristems after the irradiation treatments were rooted *in vitro*, acclimatized and moved to the greenhouse. Almost all developed mature plants showed normal morphology and flowering. After open pollination, the sterile-mutant phenotype of each floret was easily detected by an unenlarged ovary. Although a lot of inflorescences with different frequencies of sterile florets were observed at all doses tested, only branches with sterile inflorescences whose florets could produce no seeds were selected, and propagated over 3 times by cutting and growth to flowering. By repeating this selection process we could enrich the sterile phenotype by the elimination of the fertile organs of chimeric plants that were originated from irradiated meristems harboring mass proliferating cells. Finally, one mutant out of 104 FS lateral shoots irradiated at 5Gy, 1 mutant out of 115 FC shoots irradiated at 5Gy and 3 mutants out of 108 FC shoots irradiated at 10Gy were successfully isolated. In contrast, no mutant was isolated from FW. In treatments with radiations such as X-rays or gamma rays, usually, an M1 seedlings growth reduction of 30–50% or a survival rate of 40–60% compared with the control plants is used as a criterion for a promising radiation treatment (van Harten 1998). However, in the present study, sterile mutants were obtained by irradiation treatments at doses that did not inhibit lateral shoot development. These data suggest that high frequency mutation induction by heavy-ion beams is not necessarily associated with growth inhibition. In addition, in FW, one sterile mutant was isolated from ^{20}Ne-ion irradiation (2700MeV, 63 keVμm^{-1}, 1Gy) in complementary study (data not shown). This may indicate that there are varietal differences of reaction to ion species.

Table 1. Isolation of sterile mutant lines from node cultures of *V.×hybrida* after irradiation with N-ion beams[a].

Cultivars	Dose (Gy)	No of nodes irradiated[b]	No of shoots developed (%)	No of sterile mutant lines isolated
'Temari Sakura'				
(FS)	0	15	24 (80.0)	0
	1	64	107 (83.6)	0
	2	64	113 (88.3)	0
	5	64	104 (81.3)	1
	10	64	35 (27.3)	0
'Temari Coral Pink'				
(FC)	0	15	25 (83.3)	0
	1	64	115 (89.8)	0
	2	64	88 (68.8)	0
	5	64	115 (89.8)	1
	10	64	108 (84.4)	3
'Temari White'				
(FW)	0	15	27 (90.0)	0
	1	64	113 (88.3)	0
	2	64	51 (39.8)	0
	5	64	43 (33.6)	0
	10	64	0 (0.0)	0

[a] Nodes were cultured in plastic dishes and irradiated with N-ion beams.
[b] Nodes had two lateral meristems.

Characterization of flowering habits

In order to compare the flowering habits of mutants and parental cultivars, two sterile mutant lines SS and SC, which were generated from 5 Gy-irradiated FS and FC, respectively, were analyzed. We found that there are no significant differences between the morphology of florets and inflorescences of mutant and parental cultivars (Table 2). Besides, the color of florets and leaves of the mutants were the same as their parental cultivars (data not shown). Therefore, we could obtain mutants for a specific trait without diminishing the fitness of the plants.

On the other hand, the duration of inflorescence life in both mutants was very different to their parental lines. Thus, SS and SC extended their inflorescences longevities from 11.4 and 8.9 days up to 15.7 and 11.6 days, respectively (Table 2). In some species, petal senescence may occur both before and after fertilization, for example, via pollination and ethylene biosynthesis (Reid and Wu 1992; Stead 1992; van Doorn 2002). The changes in the number of inflorescences with flowering floret(s) following transplantation of plantlets to a pot are showed in Figure 1. All mutants and parental cultivars started flowering 3 weeks after the transplantation, and the number of inflorescences increased according with the growth level of the stem. The first peak of number of inflorescences appeared after 10 to 12 weeks of transplantation (Figure 1). After that, the number of

Table 2. Horticultural characterization of flowers of fertile cultivars and sterile mutants of *V.×hybrida*[a].

Line	Floret diameter (mm)[b]	Inflorescence diameter (mm)[c]	No. of florets per inflorescence[c]	Duration of inflorescences life (day)[c]	Pollen fertility (%)[d]
FS	20.5±0.3	55.6±0.7	24.8±1.4	11.4±0.5	94.7±0.7
SS	20.1±0.3	55.4±0.6	25.5±1.2	15.7±0.5	0
FC	19.0±0.2	54.4±0.9	24.3±1.4	8.9±0.6	75.2±2.1
SC	19.3±0.4	54.6±0.9	24.9±1.9	11.6±1.4	73.1±0.9

[a] Values represent the mean±standard error of at least five parental or mutants plants.
[b] Data are derived from 20 florets.
[c] Data are derived from 20 inflorescences.
[d] Pollen fertility was evaluated by aceto-carmine staining.

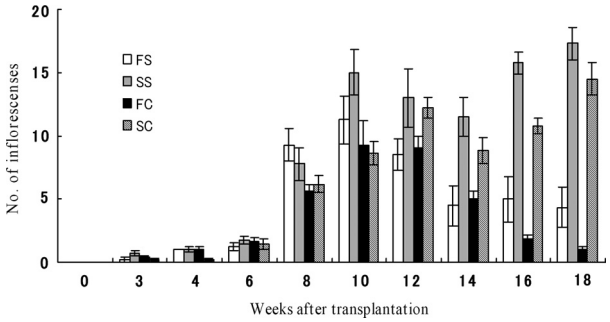

Figure 1. Changes in the number of inflorescences with blooming flowers following transplantation of plantlets to pots. Values represent the mean±standard error from four independent experiments.

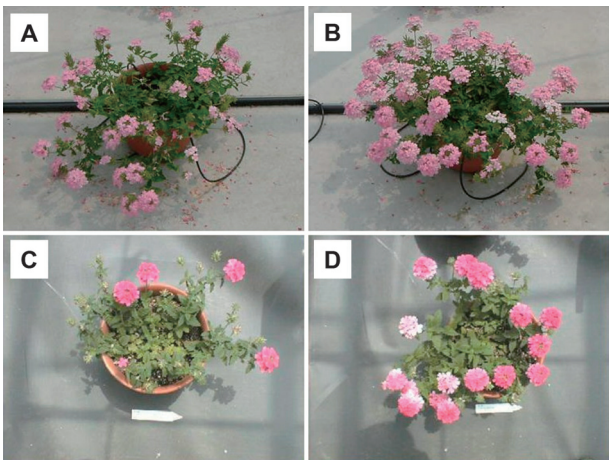

Figure. 2. Growing and flowering characteristics of fertile parental cultivars and sterile mutants in the open air. (A) FS, (B) SS, (C) FC, (D) SC

inflorescences gradually decreased. However, while the number of inflorescences continued to decrease in the parental cultivars FS and FC, it increased again in the mutant lines SS and SC. As a result, the two mutant lines presented a larger number of inflorescences compared with their parental cultivars. Leaf browning was observed in the basal part of FS and FC plants and a lot of seeds were produced on mature inflorescences. In contrast, little senescence and no seed setting were observed in SS and SC. In *Arabidopsis*, sterile mutants that do not produce seeds presented more bolts and flowers compared with their parental lines (Noodén and Penney 2001). On the other hand, it was reported that these kind of sterile mutations are associated to a prolonged Soybean life due to the production of new leaves and stems with inflorescences (Noodén 1984). Similar results were obtained in the present study. These results suggest that the attempt to produce seeds might be someway extended in time by the impossibility of mutants cannot to produce seeds. Figure 2 shows that the growing and flowering characteristics of original fertile cultivars and sterile mutants growing in the open air. There were no significant differences between sterile mutants and parental strains concerning plant width and height, resistance to climate and diseases such as root rot and powdery mildew. In contrast, the sterile mutants showed a better continuous flowering habit compared with their parental cultivars. This result indicates that the sterile mutants keep not only the horticultural merits of their parental cultivars but also additional advantages.

Characterization of the sterile phenotype

Pollen fertility at anthesis was assessed by aceto-carmine staining (Table 2). All stained pollens showed homogeneous morphology. All SS pollens aborted and therefore were not stained by aceto-carmine. In contrast, most of the FS pollens (94.7%) were stained, which indicates that they are fertile. FC and SC have similar levels of fertile pollens (75.2% for FC, 73.1% for SC). These results indicate that while SC mutant has viable pollens, SS mutant has pollens that are completely sterile. The SS mutant is likely defective in 1 or more essential gene involved in pollen maturation, but further investigation of pollens and female gametes were needed.

In order to investigate the fertility of pollens and embryo sacs, we performed pollination tests between mutant lines and their corresponding parental cultivars. Seed set following self- and cross-pollinations is shown in Table 3. Seed set of SS was not observed in either self-pollinations or crossing tests, which indicates that both female and male gametes are sterile. On the other hand, the fact that cross pollinations of SC×FS and SC×FC plants produced as many seeds as selfing of FS and FC indicates that SC has fertile embryo sacs. However, in the selfing of SC, FS×SC and FC×SC plants a very low seed setting was observed. For further determination of SC pollen fertility, SC pollens were crossed with FW and FL florets which have functional female gametes. As a result, it was observed that FW×SC and FL×SC plants could produce seeds normally, although the seed setting rates were lower than those of SC×FS, SC×FC and so on. This fact indicates that SC pollens are functional. *V.×hybrida* is thought to be derived from a multiple hybrid involving *V. incisa*, *V. peruviana*, *V. phlogiflora* and *V. teucroides* (Griffiths 1994). The slightly low seed setting rates of FW×SC and FL×SC may be caused by some differences of cross compatibility among the varieties that arise from the repeating complicate crossings and/or inbreeding in the long breeding history. On the other hand, few self-pollinated flowers could produce seeds. Therefore, next we proceeded to analyze the pollen-pistil interaction in self-pollinated FC and SC plants in order to determine the post-pollination mechanism involved in the SC mutant phenotype.

In both self-pollinated FC and SC, a lot of pollen tubes grew into the pistils 3 h after selfing (Figure 3A, B). However, while self-pollen tubes of FC penetrated

Table 3. Seed-set following self- and cross-pollination of *V.×hybrida* cultivars and sterile mutants.

♀	Pollination	♂ FS	SS	FC	SC
FS	Non-controlled[a]	230/366[c] (62.8)[d]	—	—	—
	Controlled[b]	71/123 (57.7)	0/120 (0)	62/101 (61.4)	2/114 (1.8)
SS	Non-controlled	—	0/264 (0)	—	—
	Controlled	0/115 (0)	0/58 (0)	0/98 (0)	0/106 (0)
FC	Non-controlled	—	—	216/228 (94.7)	—
	Controlled	54/102 (52.9)	0/98 (0)	58/96 (60.4)	1/109 (0.9)
SC	Non-controlled	—	—	—	2/256 (1.2)
	Controlled	75/121 (62.0)	0/87 (0)	50/107 (46.7)	1/156 (0.6)
FW	Controlled	—	—	—	20/109 (17.2)
FL	Controlled	—	—	—	28/103 (27.2)

[a] Non-controlled self-pollination.
[b] Controlled pollination with emasculated florets as indicated in materials and methods.
[c] No. of florets producing seeds/No. of florets pollinated
[d] Percentage of florets producing seeds

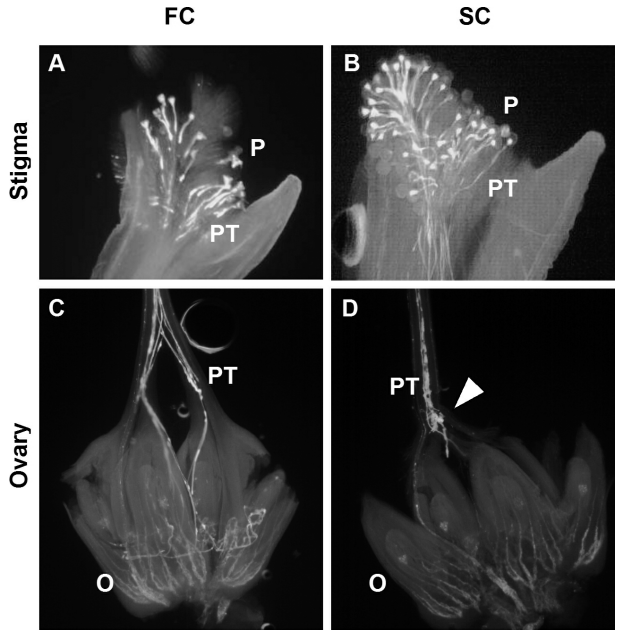

Figure. 3. Fluorescence micrographs of FC and SC pistils following self-pollination. (A, B) Germinating pollens on surface of stigma 3 h after selfing. (C, D) Pollen tube growth in styles and ovaries 24 h after selfing. P, pollens. PT, pollen tubes. O, ovules. Arrow head shows abnormal growth of pollen tubes. Aniline blue staining fluorescence was observed by an UV light fluorescence microscope.

into four ovaries 24 h after selfing (Figure 3C), most of the SC pollen tubes accumulated at the lower part of the style or the upper region of the ovary (arrow head), and ovules remained unfertilized (Figure 3D). These results indicate that SC exhibits self-incompatibility. Self-incompatibility may occur in the stigma, style or ovary. Among them, the inhibition of incompatible pollen tubes at the lower part of the style or at the ovary has been described as late-acting self-incompatibility (Seavey and Bawa 1986). In teak (*Tectonia grandis* Linn. f.), which belongs to the Verbenaceae family, a self-incompatibility phenotype similar to SC has been reported (Tangmitcharoen and Owens 1997a). In teak, the site of the final pollen tube arrest was generally the ovary, and most of the pollen tubes did not enter the embryo sacs. The very low level of seed production after selfing teak (2.5%) (Tangmitcharoen and Owens 1997b) and SC (about 1%) might be explained by unusual fertilization such as bud pollination or delayed pollination overcoming self-incompatibility (Shivanna 2003). The cross-pollinations FS×SC and FC×SC could produce only a few seeds. These results suggest that SC pollens are incompatible with the pistils of FS and FC. The results from the analysis of pollen-pistil interaction in SC and the previous reports about self-incompatibility in teak (Tangmitcharoen and Owens 1997a,b) suggest that the sterile mutant SC may be a revertant that regained the self-incompatibility of ancestral wild species of *Verbena×hybrida*. However, at the present time, there are no reports confirming the existence of self-incompatible species in wild type *Verbena* species. A detailed characterization of the self-incompatibility phenotype in the genus *Verbena* is in progress.

In the present study, we reported the isolation of sterile mutants at high frequency by heavy-ion beam irradiation. Two of the sterile mutants, SS and SC, had more flower clusters than the parental cultivars. Their elite clones of SS and SC were released to the market as new ‘Temari Sakura’ and ‘Temari Coral Pink’ in 2002 and 2003, respectively. It was the first success in the world to commercialize new cultivars produced by using heavy-ion beams. We can conclude that heavy-ion beam irradiation is an excellent tool for high frequency mutation breeding of sterile mutants. In addition, inducible male sterility technique will be useful for transgenic plants to prevent transgene flow through pollen dispersal.

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