

High-efficiency improvement of transgenic torenia flowers by ion beam irradiation

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Received November 6, 2007; accepted December 7, 2007 (Edited by H. Tsujimoto)

Abstract To shorten the time required for breeding and optimize the risk-cost/benefit ratio of genetically modified ornamental plants, we applied heavy-ion beam irradiation to wild-type and genetically modified torenia (*Torenia fournieri* Lind. CV. 'Crown Violet' plants in which petal color and pattern had been modified by controlling two anthocyanin biosynthesis-related genes encoding chalcone synthase (*CHS*) and dihydroflavonol-4-reductase (*DFR*). Ion beams of ¹²C⁶⁺ and ²⁰Ne¹⁰⁺ were applied to 11,500 leaf disks from wild type and five transgenic lines, and over 3,200 regenerated flowering plants were then investigated for visible phenotypes. The mutation rate after whole irradiation averaged 10.4%, and the maximum rate in the initial screening was 44.2% (²⁰Ne, 30 Gy). Mutant phenotypes were observed mainly in flowers and showed wide variation in color and shape. Mutation efficiencies for petal color and coloration pattern were higher in transgenic plants than in wild-type plants, while those for petal shape and corolla divergence were almost equivalent in the two plant groups. Mutation spectrums in petal color in transformant-based mutants were obviously wider than those in wild-type plants. Among these mutants, a class B gene-deficient mutant was investigated as a model case for further study to facilitate the control of flower phenotype. Expression of the *TfGLO* gene was found to be repressed in this line, probably due to dysfunctioning of the upstream signaling. We propose that the combination of genetic engineering and ion beam irradiation greatly facilitates improvement of agrobiological and commercial traits within a short period. We also discuss characteristic changes observed at high frequency in torenia flowers and the mutant-based approach to the identification of useful genes.

Key words: Anthocyanin, class B genes, ion beam breeding, petal coloration, transgenic torenia.

Recent progress in plant genetic engineering has led to the production of genetically modified crops with many valuable properties that traditional breeding methods have not been able to produce. Examples include vitamin A-enriched rice (Ye et al. 2000), herbicide-resistant soybean (Delannay et al. 1995; Padgett et al. 1995), virus-resistant papaya (for review; Gonsalves 1998), and blue petals in carnations and roses (Fukui et al. 2003; Katsumoto et al. 2007). Some of these genetically modified organisms play important roles in modern life and have commercial applications, but many others are still not suitable for release because of the high costs of risk assessment and their adaptability to the Cartagena Protocol domestic law (Watanabe et al. 2005; Chandler and Tanaka 2007).

Ion beam breeding has now become a routine procedure, and many useful traits have been added to commercial crops, especially flower crops. Since ion

beam irradiation, in contrast to other mutagenic techniques, efficiently changes target phenotypes and confer a broad spectrum of new traits without affecting other agronomical traits (Shikazono et al. 2005); the resulting products are available for use after a relatively short breeding period. For example, plants modified by this method that are now commercially used in Japan include verbena with an increased number of inflorescences (Saito et al. in this issue), chrysanthemums with various colors and reduced axillary bud formation, torenias with altered petal color, and buckwheat with a dwarf phenotype.

Although genetic engineering and ion beam breeding have beneficial features, both still have practical or technical disadvantages in the production and commercialization of transgenic plants on demand. For example, public release of genetically engineering products may experience problems such as cost of

Abbreviations: AP1, APETALA1; AP3, APETALA3; CHS, chalcone synthase; DFR, dihydroflavonol-4-reductase; LFY, LEAFY; PI, PISTILATA; TFACT3, torenia actin3; TfDEF, torenia DEFICIENS; TfGLO, torenia GLOBOSA; UFO, unusual floral organs.

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

commercialization and overcoming public resistance. Likewise, ion beam breeding is hampered by low reproducibility—it is difficult to obtain the same mutant in different experiments. Such deficiencies might be compensated in part by combining these procedures thereby improving the risk-cost/benefit ratio and to identify the genes that produce valuable mutant phenotypes.

Torenia, an annual plant of the figwort family, is a useful material for genetic engineering because of its simplicity and small genome size ($2n=18$, 171 Mbp) (Kikuchi et al. 2006), its amenability to tissue culture and transformation (Aida et al. 2000), short generation time, and small plant size. The simplicity of the structure and color pattern of *torenia* flowers confers a considerable advantage for the detection of slight changes in phenotype. Normal blooming under high humidity (e.g. in plant boxes) and an elastic photoperiod (at least 8–16 h) help reduce the excess space and effort needed for maintenance. *Torenia* is also known as a useful material for studying pollen tube guidance because it has an exposed ovule (Higashiyama et al. 1998). Therefore, *torenia* is now regarded as a useful plant model for studying the phenotypes of flowers such as the snapdragon and petunia.

To develop an efficient way to produce and release transgenic flowers to the public domain, as well as to improve the risk-cost/benefit ratio of product commercialization, we performed ion beam irradiation of wild-type and genetically modified *torenia* plants.

This study provides not only a model case for an efficient variety and value-adding system for transgenic crop plants but also good material for subsequent breeding and molecular study.

Materials and methods

Plant materials

Torenia fournieri Lind. CV. ‘Crown Violet’ was used as a wild-type (WT), non-transgenic control plant (Figure 1A). Petal color-changed transformants based on this lineage have been reported previously (Aida et al. 2000). Five transgenic lines with distinct petal colors and coloration patterns were selected and subjected to irradiation. The transgenic line CS400-29 (Figure 1B) contains a transgene encoding *torenia* chalcone synthase gene (*CHS*) under the control of cauliflower mosaic virus 35S RNA promoter (p35S). Similarly, CA411-3 and CA411-7 contain p35S::antisense-*CHS* (Figure 1B), DS405-2 contains p35S::*DFR* (*DFR* is dihydroflavonol-4-reductase) and DA416-20 contains a p35S::antisense-*DFR* gene (Figure 1C). Note that each line number is prefixed with letters designating the introduced gene construct (for example, CS means *CHS*-sense). This information was not given in the previous report (Aida et al. 2000). Petal colors in these lines have been modified, as shown in Figure 1, by co-suppression or the antisense effect of the introduced genes. Plant materials were maintained in a plant box supplemented with 1/2 Murashige and Skoog (MS) medium containing 0.2% gellan gum and reproduced vegetatively by herbaceous cutting.

Ion beam irradiation

Leaf disks (5 mm square) were prepared from *in vitro* grown

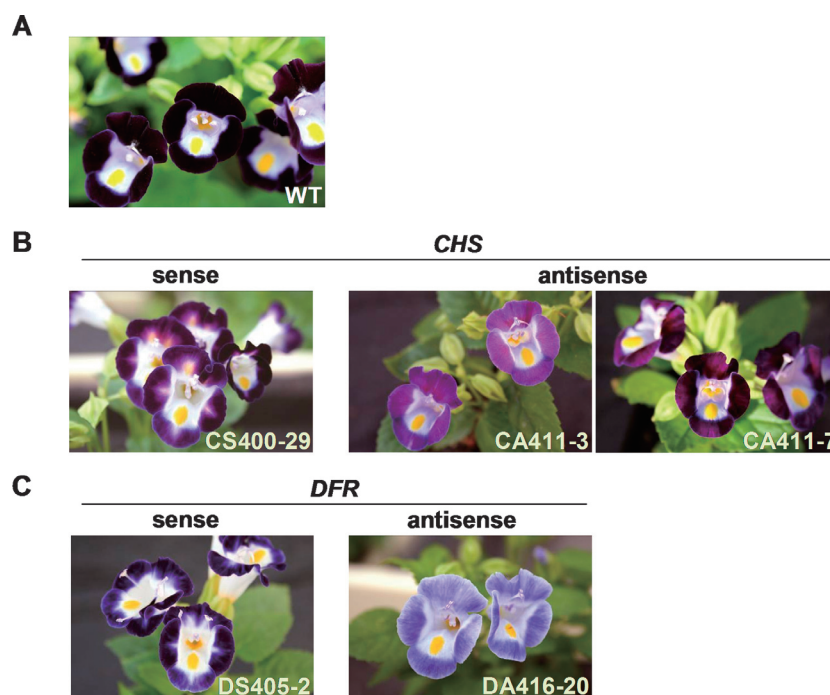


Figure 1. Transgenic *torenia* plants used for ion beam irradiation. (A) Photograph of wild-type (WT) *torenia* plant. (B) Photographs of transgenic *torenia* carrying p35S::*CHS* (CS400-29) and p35S::antisense-*CHS* (CA411-3 and CA411-7). (C) Photographs of transgenic *torenia* carrying p35S::*DFR* (DS405-2) and p35S::antisense-*DFR* (DA416-20).

torenia plants 5–7 cm in height, and placed on 9 cm plates supplemented with 1/2 MS-0.2% gellan gum at 80–100 disks per plate. To equalize the physiological conditions of the leaf disks in each plate, leaf disks were prepared from two to four independently grown plantlets. At least two plates were used for each type of irradiation. Ion beams of ^{12}C (1.62 GeV, LET 22.5 KeV μm^{-1} , 5–80 Gy) and ^{20}Ne (2.70 GeV, LET 61.5 KeV μm^{-1} , 5–30 Gy) were applied at the E5 facility of the RIKEN RI-beam factory (RIBF) using automatic irradiation control and a sample changer system (Ryuto *et al.* in this issue). After irradiation, leaf disks were transferred to shoot-inducing plates supplemented with MS-0.2% gellan gum containing 1 mg l^{-1} 6-benzylaminopurine (BA) (40 disks per plate) and incubated at 25°C under fluorescent light (16L/8D, 160 $\mu\text{mol m}^{-2} \text{s}^{-1}$) to induce adventitious shoots.

Regeneration and vegetative reproduction

Forty days after irradiation, adventitious shoots 1 cm in height were collected and transferred to 1/2 MS plant boxes to promote rooting and subsequent growth of plantlets. Up to 70 shoots were collected to obtain 50 flowering plants for each type of irradiation. To prevent the duplication of transgenic plants that originated from single cells, only one shoot was taken from a leaf disk. Herbaceous cuttings were taken from plantlets 3–5 cm in height and placed on an imbibed sphagnum peat pellet for rooting (Jiffy 7, Jiffy Products International AS) and acclimatized in a humidified incubator (Type 513A; Ozawa Inc.) for 4 weeks. Plantlets that showed normal root formation and growth were transferred to 7.5 cm vinyl pots (TO Poly Pot; Tokai Agrisystem) containing conditioned soil consisting of fertilized granulated soil (Kureha) and MetroMix 350 (Sun Glo Horticulture Distribution Inc.) at a volume ratio of 50:50, and grown in an air-conditioned contained greenhouse under natural daylight.

Observation of phenotypes and evaluation of their stability through vegetative reproduction

Thirty days after potting, flower phenotypes were observed on petal color, coloration pattern, petal shape, corolla divergence, and pollen development. Phenotypes that resulted in a reduction in plant growth or viability, such as dwarf, albino, and fasciated shoot, were eliminated from the investigation because we thought that the lethality may impose difficulties in determining the true mutation rates for these plants. Also, the first 10 blooms of each plant were excluded from analysis because the flower phenotypes at the beginning of the flowering period are sometimes unstable, especially with regard to petal number and shape, although such instability seems a natural feature in the torenia plant (data not shown). Plants with at least one normal flower were evaluated as representing a normal phenotype, except when the phenotype was clearly chimeric. For the first screening, mutated phenotypes were confirmed using at least 10 flowers per observation; candidates were then reconfirmed twice using the continuously maintained original plants at an interval of 1 month. Pollen development was confirmed visually by rubbing mature anthers against petals; viability and fertility were not tested.

For the second screening, phenotypic stability in putative mutants selected by the first screening was evaluated using

herbaceously propagated plants for at least two different reproductions, using the observation procedure used in the first screening.

RT-PCR and genomic PCR analysis

Total RNA was prepared from whorls 1, 2, and 4 of mature floral buds of three independent WT, DA416-20 and 53-31 plants by RNeasy (Qiagen). cDNAs were synthesized from the total RNAs with a cDNA Synthesis Kit (Stratagene), and used to amplify the transcripts. The RT-PCR reaction was performed using KOD Plus 2 (Toyobo). The sequence of each specific primer is described in Table 3. The quality and quantity of each cDNA sample were checked using the *TjACT3* gene (AB330989) as an internal control.

Torenia genomic DNA was prepared from leaves using Isoplant II (Nippon Gene Co., Ltd.). The 1.4 kb promoter region of the *TjGLO* gene was isolated using a GenomeWalker kit (Clontech) according to the manufacturer's instructions, using *TjGLO* gene-specific first (5'-ACACGAGCATCACAGAGAACTACTAATCTCC-3') and second (5'-TAAGTAACCTGCCTGTTGCTTGTAGTTTTCG-3') primers.

Results

Mutation rates in the first screening

Initial mutation rates in ion beam irradiated plants are shown in Table 1 (Mutated, Initial). Of the 3,241 flowered plants, 338 (10.4%) showed altered phenotypes. The mutation rate increased with increasing irradiation dose in most cases. The pattern of increase differed between the two ion species. For ^{12}C irradiations, the mutation rate started to increase at 35 Gy and showed a gradual increase until 80 Gy, while ^{20}Ne irradiations tended to peak sharply at 30 Gy. The highest rate (44.2%) was calculated for irradiation at ^{20}Ne -30 Gy in the *DFR*-antisense line, DA416-20. The total mutation rate in ^{12}C -irradiated plants (11.7%) was higher than that in ^{20}Ne -irradiated plants (9.4%).

Phenotypic consistency under vegetative reproduction

Some of the phenotypes observed during the first screening seemed unstable and were lost during subsequent vegetative reproduction. We therefore decided to treat only the stable transformants as actual mutants thereafter. To select such stable mutants and determine the efficiency of stable mutation, we examined the constancy of the phenotypes using at least two independent vegetatively reproduced plants for each mutant. As shown in Table 1, 171 of 338 initial mutants were stable in their phenotypes (Table 1; Mutated, Stable) and total consistency was calculated at 50.6%. The distinctive patterns of dose-dependent increase in mutation efficiency observed in the initial screening were basically unchanged, although some were obscured by the diminished number of candidates.

Table 1. Mutation rates of ion-beam irradiated plants after the first screening and vegetative reproductions (second screening).

Original lines	¹² C						²⁰ Ne					
	Dose (Gy)	No. plants	Mutated				Dose (Gy)	No. plants	Mutated			
			Initial		Stable				Initial		Stable	
			No.	Rate (%)	No.	Rate (%)			No.	Rate (%)	No.	Rate (%)
WT (control)	5	69	0	0.0	0	0.0	5	70	0	0.0	0	0.0
	20	73	1	1.4	0	0.0	10	81	2	2.5	1	1.2
	35	74	7	9.5	4	5.4	15	72	4	5.6	4	5.6
	50	77	7	9.1	4	5.2	20	63	3	4.8	1	1.6
	65	71	9	12.7	4	5.6	25	77	7	9.1	3	3.9
	80	34	3	8.8	1	2.9	30	57	6	10.5	1	1.8
CS400-29	5	nt	—	—	—	—	5	nt	—	—	—	—
	20	55	3	5.5	1	1.8	10	60	3	5.0	0	0.0
	35	nt	—	—	—	—	15	52	3	5.8	2	3.8
	50	47	9	19.1	8	17.0	20	58	3	5.2	2	3.4
	65	nt	—	—	—	—	25	55	7	12.7	6	10.9
	80	40	7	17.5	6	15.0	30	27	6	22.2	3	11.1
CA411-3	5	46	3	6.5	1	2.2	5	46	0	0.0	0	0.0
	20	31	0	0.0	0	0.0	10	41	1	2.4	1	2.4
	35	66	7	10.6	5	7.6	15	52	2	3.8	0	0.0
	50	41	4	9.8	2	4.9	20	58	7	12.1	4	6.9
	65	33	9	27.3	5	15.2	25	16	3	18.8	1	6.3
	80	35	12	34.3	6	17.1	30	42	7	16.7	2	4.8
CA411-7	5	59	0	0.0	0	0.0	5	55	2	3.6	1	1.8
	20	68	2	2.9	1	1.5	10	53	5	9.4	1	1.9
	35	69	10	14.5	4	5.8	15	21	0	0.0	0	0.0
	50	46	4	8.7	2	4.3	20	68	5	7.4	5	7.4
	65	34	6	17.6	3	8.8	25	53	4	7.5	4	7.5
	80	38	8	21.1	3	7.9	30	40	5	12.5	2	5.0
DS405-2	5	nt	—	—	—	—	5	nt	—	—	—	—
	20	33	3	9.1	2	6.1	10	57	3	5.3	2	3.5
	35	nt	—	—	—	—	15	38	2	5.3	0	0.0
	50	49	9	18.4	5	10.2	20	60	7	11.7	3	5.0
	65	nt	—	—	—	—	25	56	2	3.6	0	0.0
	80	35	10	28.6	8	22.9	30	36	11	30.6	6	16.7
DA416-20	5	43	1	2.3	0	0.0	5	56	2	3.6	1	1.8
	20	46	3	6.5	2	4.3	10	56	9	16.1	3	5.4
	35	48	4	8.3	2	4.2	15	41	7	17.1	1	2.4
	50	41	14	34.1	4	9.8	20	53	10	18.9	5	9.4
	65	29	5	17.2	3	10.3	25	46	8	17.4	5	10.9
	80	52	13	25.0	8	15.4	30	43	19	44.2	7	16.3
Subtotal	WT	398	27	6.8	13	3.3	WT	420	22	5.2	10	2.4
	TG	1084	146	13.5	81	7.5	TG	1339	143	10.7	67	5.0
Total	WT	818	49	6.0	23	2.8						
	TG	2423	289	11.9	148	6.1						

Total numbers of mutants and average mutation rates were compared between the wild type-based mutants (WT) and the transformant-based mutants (TG). nt: not tested, —: no datum

Variations in flower phenotypes induced by irradiation

Typical flower mutants that stably maintained their phenotypes after vegetative reproduction are shown in Figure 2. Mutant phenotypes showed a wide range of color and coloration pattern, including tone-shifted (29-17, Figure 2B; 45-32, Figure 2D), bordered (2-47, Figure 2B; 45-18, Figure 2D), gradated (2-20, Figure 2B; 15-64, Figure 2D), streaked (12-6, Figure 2C; 47-10, Figure 2D), tie-dyed (40-53, Figure 2C; 16-19, Figure 2D), and

other patterns. Most of these might be explained by changes in anthocyanin composition and content in various parts of the petals. As seen in the cases of 2-21 and 27-18 (Figure 2B), the change in petal coloration pattern sometimes differed within and between petals, suggesting differences in the regulation of pigmentation between the three petal positions (upper, lateral, and lower) and regions in each petal. Changes in carotenoid content in each petal (10-4, 39-46, Figure 2C) and blotch (a large yellow dot in the lower petal) size, shape and

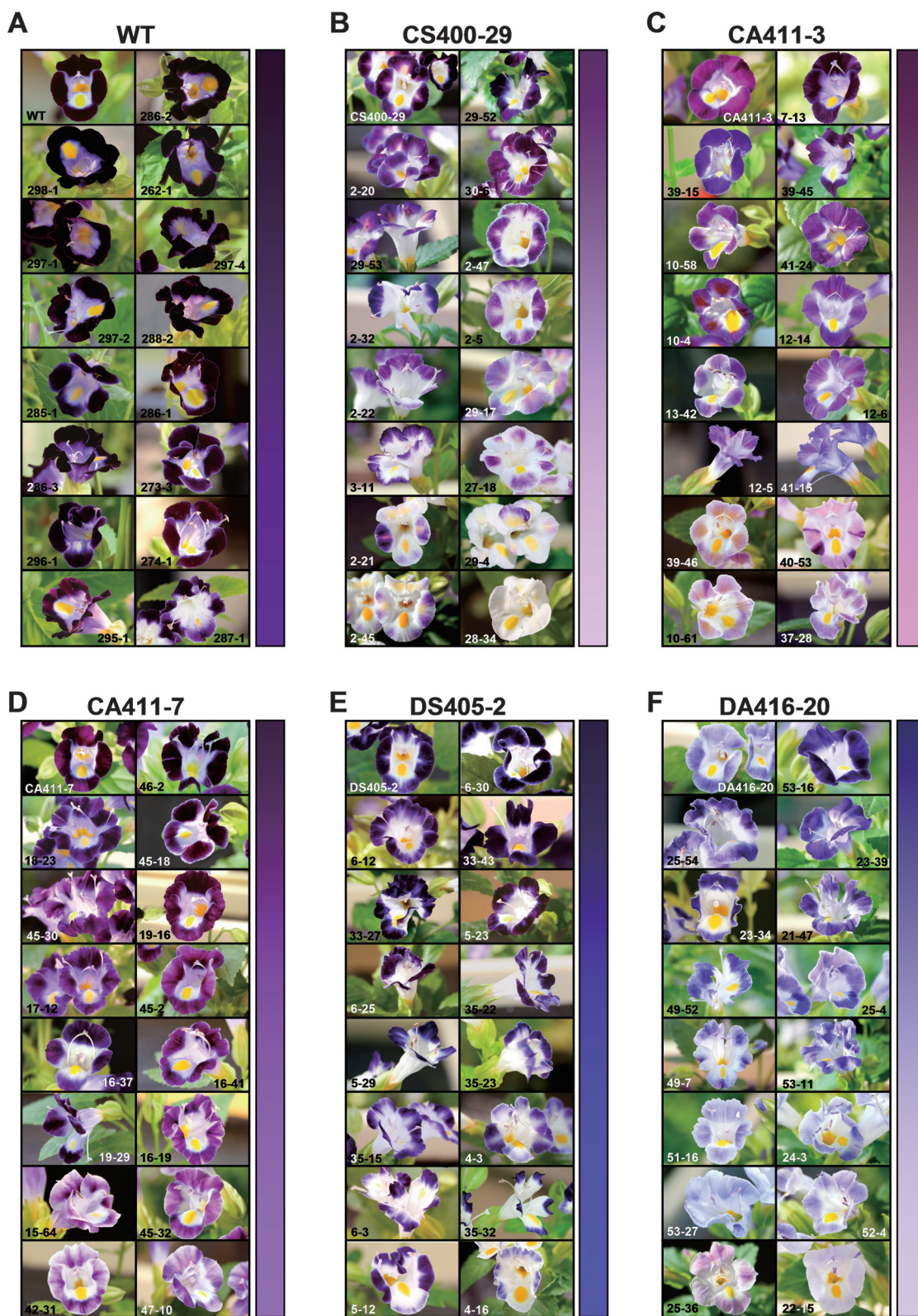


Figure 2. Morphological and color variations induced by ion beam irradiation. Photographs of mutants isolated by ion beam irradiation of WT plants (A), CS400-29 (B), CA411-3 (C), CA411-7 (D), DS405-2 (E), and DA416-20 (F). The original flower of each plant material is shown in the top-left corner of the column. Fifteen distinctive mutants selected from each original plant are sequenced by color depth from top to bottom. The line number of each mutant is indicated in the photograph. A representation of the mutation spectrum in each irradiation experiment is shown as a color chart beside the column.

tone (296-1, Figure 2A; 4-16, Figure 2E) also contributed, especially in combination with the change in anthocyanin content.

In addition to these coloration-based phenotypes, changes in morphological traits strongly affected the appearance of flowers. Such changes included petal shape and corolla divergence, such as rounded (2-21, Figure 2B; 16-37, Figure 2D), angulated (17-12, Figure 2D; 25-4, Figure 2F), wavy (23-34, 25-54, Figure 2F), bent (2-32, Figure 2B; 45-30, Figure 2D), fringed (3-11, Figure 2B, 51-16, Figure 2F), dissociated (41-15, Figure 2C, 19-29, Figure 2D), and less divergent (29-53, Figure 2B; 35-23, Figure 2E). These phenotypes, except for fringed petals, were also abundant and equal in number to the flower color mutations, and enabled us to clearly distinguish the mutants from the original plants. Combinations of coloration-based and morphological mutations might also contribute to the increased variation in flowers.

Wide-range mutation spectrum in transgenic plants

Flower colors in the WT-based mutants (Figure 2A) seemed less varied than those in the transgenic-based mutants (Figure 2B–F). It is possible that the higher mutation efficiency in transgenic plants depends on their wider variation in petal color, which would be affected by the *CHS* or *DFR* transgenes. We therefore compared the proportion of mutant phenotypes between coloration-based mutations and morphological mutations in WT-based mutants and transformant-based mutants. As shown in Table 2, changes in petal colors and coloration patterns in the WT-derived mutants seemed less abundant compared with the transformant-derived mutants, especially in the irradiation of ^{12}C (Group 1), while no significant changes were observed in petal shape and corolla divergence (Group 2). This difference in petal color mutation efficiency may in part explain the reduction in total mutation efficiency in WT plants, as indicated in Table 1.

Analyses of B-function-deficient plants

As shown in Figure 2, the combination of genetic engineering and ion beam breeding appeared to be advantageous in the production of plants with valuable

traits. This combination could be used to identify the genes providing such useful phenotypes in mutants as well as to elucidate the mechanisms of this process.

Among the mutants obtained by heavy ion beam irradiation, we chose a floral phenotype mutant for further analyses, because the molecular and/or genetic mechanisms of floral developmental control are poorly understood in *torenia*. The 53-31 mutant, which was obtained by the ^{20}Ne (30 Gy) ion beam irradiation of DS416-20, exhibited a B-function-deficient phenotype in which the second whorls (Figure 3Ad, WT) were changed into sepals (Figure 3Ae, f), as seen in snapdragon (Tröbner et al. 1992) and *Arabidopsis* (Bowman et al. 1989, 1991). We assumed that the *torenia* class B genes *GLOBOSA* (*TfGLO*) and/or *DEFICIENS* (*TfDEF*), which are orthologs of *PISTILATA* (*PI*) and *APETALA3* (*AP3*) in *Arabidopsis*, respectively, would be deficient in the 53-31 mutant. Then, we examined the expression of the *TfGLO* and *TfDEF* genes using the first, second and fourth whorls of the mutant, by RT-PCR analysis (Figure 3B). The results indicated that only *TfGLO* expression was not detected after 28 cycles of RT-PCR in all whorls. Expression of *TfGLO* was

Table 2. Comparison of phenotype-specific mutation rates.

Ion species	Original line	No. mutated	Mutation			
			Group 1		Group 2	
			No.	Rate (%)	No.	Rate (%)
^{12}C	WT	13	5	38.5	12	92.3
	CS400-29	15	14	93.3	9	60.0
	CA411-3	19	18	94.7	17	89.5
	CA411-7	13	10	76.9	7	53.8
	DS405-2	15	14	93.3	15	100.0
	DA416-20	19	17	89.5	14	73.7
	Subtotal	94	78	83.0	74	78.7
^{20}Ne	WT	10	5	50.0	9	90.0
	CS400-29	13	12	92.3	9	69.2
	CA411-3	8	6	75.0	6	75.0
	CA411-7	13	12	92.3	8	61.5
	DS405-2	11	6	54.5	11	100.0
	DA416-20	22	17	77.3	19	86.4
	Subtotal	77	58	75.3	62	80.5
Total	171	136	79.5	136	79.5	

Group 1: Mutations in petal color and coloration pattern
Group 2: Mutations in petal shape and corolla divergence

Table 3. Primer sequences for RT-PCR

Gene name	Gene accession	Direction	Primer sequence
<i>TfGLO</i>	AB359952	Forward	5'-CTTAAAAACGGAATGTCAGC-3'
		Reverse	5'-GACAGGTTTCGAAATTATAATTGGT-3'
<i>TfDEF</i>	AB359951	Forward	5'-ATGGCTCGTGGTAAGATACAGATC-3'
		Reverse	5'-CACACAAACATACCAACCACATTT-3'
<i>TfACT3</i>	AB330989	Forward	5'-AAATACAGTGTGGATCGGAGGTTTC-3'
		Reverse	5'-GAATAGCACACAGAGAATAGCAAACC-3'

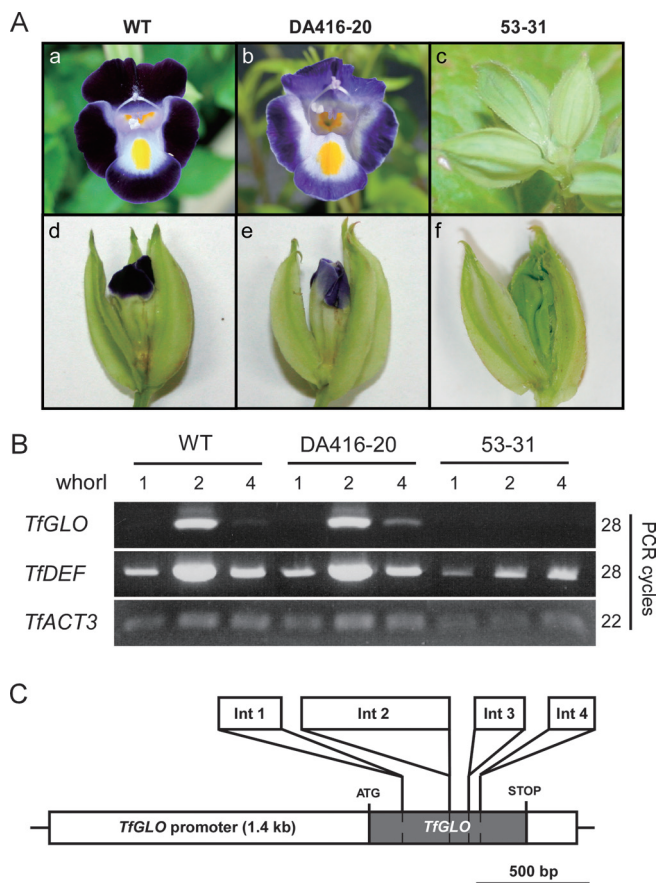


Figure 3. Analysis of mutants obtained by heavy ion beam irradiation. (A) Photographs of WT (a), DA416-20 transgenic line (b) and 53-31 mutants (c) obtained from the DA416-20 line. Photographs of second whorls in WT (d), DA416-20 (e), and 53-31 mutants (f). (B) RT-PCR analysis of class B genes *TjGLO* and *TjDEF* in whorls 1, 2, and 4 of WT, DA416-20 and 53-31 plants. The torenia actin gene, *TjACT3*, was used as an internal control. (C) Genomic structure of *TjGLO* gene. ATG, initiation codon. STOP, stop codon. Gray box indicates the coding region of *TjGLO* (693 bp). The lengths of the introns are in the order, 135, 662, 212, and 199 bp.

detected after 22 cycles in WT and DA416-20 plants, but was not determined even after 30 cycles in the mutant (data not shown). To investigate whether this mutant phenotype was caused by a mutation in the *TjGLO* gene, we next determined the genomic sequence of *TjGLO* and its 1.4 kb promoter region in WT plants. Comparison of the 693 bp *TjGLO* cDNA and the genomic sequence showed that *TjGLO* has five exons and four introns (Figure 3C). The introns are 135, 662, 212 and 199 bp long. Consequently, specific mutation was not seen in the genomic sequence of *TjGLO* in the 53-31 mutant, even after the 1.4 kb promoter region had also been sequenced (data not shown).

Discussion

Average mutation rates in ^{12}C -irradiated plants were mostly higher than in ^{20}Ne -irradiated plants under the

conditions used in this study. Although this result led us to expect more efficient mutation with higher doses, especially for ^{20}Ne , our preliminary experiment in which WT plants were subjected to over dosage of irradiation (up to 120 Gy for ^{12}C and 40 Gy for ^{20}Ne) resulted in a reduction in adventitious shoot formation and the induction of severe phenotypes, such as strong deformation of the whole plant and retardation of flowering (data not shown). This observation, combined with the general dose-dependence of the accumulation of unfavorable mutations, leads us to consider that the use of high-dose irradiation might be inappropriate in this case. The optimum irradiation dose for efficient mutation, without affecting viability and/or other important traits, might be 50–80 Gy for ^{12}C and 20–30 Gy for ^{20}Ne . Previous work in which the optimum irradiation dose was reported to 20 Gy of ^{20}Ne (Miyazaki et al. 2006) and 50 Gy of ^{14}N (Kanaya et al. 2006) for *Torenia hybrida* cv. ‘Summer Wave Blue’ supports this idea. On the other hand, the mutation rate in ^{20}Ne -irradiated plants exhibited a relatively sharp increase in the range of 20–30 Gy, while that in ^{12}C -irradiated plants showed a dose-dependent gradual increase. This is intriguing in that it suggests a difference between the two ion species in the location of a “sweet spot”.

Investigation of phenotypic stability through vegetative reproduction revealed that half of the initially observed phenotypes were unstable (Table 1). The difference between the initial mutation rates and the stable mutation rates after the second screening suggests plurality of the mechanisms underlying changes in phenotype. It is probable that most of the stable phenotypic changes induced by ion beam irradiation were caused by the direct modification of endogenous genes closely related to the altered phenotypes, while unstable phenotypic changes might be induced by some indirect effect, such as a physiological change in the cells or an altered response to environmental conditions. To evaluate further the stability of the observed phenotypic changes in petal colors, we are planning to examine flavonol content and the expression of genes related to anthocyanin biosynthesis. Confirmation of phenotypic stability through reproductive fertilization is also under way. These studies would also help us to understand how direct the ion beam works to change phenotypes.

Mutation efficiencies in the transgenic plants even after the second screening were too high to be explained by direct disruption of introduced genes or of anthocyanin biosynthesis-related genes, resulting from genomic alterations caused by the ion beam. We think that there are four possible explanations for this high frequency of phenotypic changes in flowers: (1) the high visibility of phenotypic change in the torenia flower because of the simplicity of its floral structure; (2) involvement of multiple factors in the regulation of petal

color and shape (as mentioned in the next paragraph) as well as variation within and between petals; (3) high viability of nonreproductive floral organ mutants; and (4) instability and/or sensitivity to ion beams of the introduced *CHS* and *DFR* genes. The first two of these possibilities are the most plausible and are supported by our results shown in Figure 2. The third possibility is supported by the fact that we found no reproductive organ-defective mutant, although a few candidates exhibiting reduced-pollen phenotypes were observed. The fourth possibility cannot be rejected because mutation efficiencies in transgenic plants were higher than those in WT plants (Table 1). It is possible that frequent phenotypic change in petal color (Table 2; Group 1) reflects an unstable state of introduced genes related to anthocyanin biosynthesis. Frequent phenotypic changes in petal color might reflect an unusual genomic configuration of introduced anthocyanin biosynthesis-related genes that causes gene silencing, such as epigenetic control including methylation of the promoter sequence of the transgene or unexpected suppression of gene expression by the incidental formation of RNAi-inducing sequences in transgenes (Kanazawa et al. 2007; Fojtová et al. 2006). In addition, in these transgenic plants there would be many more opportunities than in WT plants for such genomic changes to occur with ion beam irradiation, not only in endogenous genes (Muskens et al. 2000) but also in transgenes. This notion would also explain the high mutation efficiency in transgenic-based mutants.

As observed in Figure 2, all transgenic plant-based mutants showed wider variation in color than WT-based mutants. High-efficiency mutation in transgenic plants might also be explained by the difference in the initial anthocyanin content of the starting materials between WT (Figure 1A) and transgenic plants (Figure 1B, C). Wild-type torenia has a dark color and a flat pattern in the pigmentation area of the petals, and it is difficult to detect slight changes in color and pattern after irradiation. The light colors and the coloration patterns of the petals of transgenic plants make changes more perceptible. Anthocyanin-dependent petal pigmentation is not controlled only by the composition and functionality of catalytic enzymes related to flavonoid biosynthesis. It has been demonstrated that three additional petal color determinants are concerned in this process: (1) intra- and intermolecular complex formation involving anthocyanin alone or with the copigments, flavones and flavonols; (2) the formation of metal complexes within the pigment molecules; and (3) vacuolar pH (Brouillard and Dangles 1994). Epidermal cell shape has also been reported to participate in petal color expression in snapdragon (Noda et al. 1994). In addition, multidimensional recognition of petal color patterns enables us to distinguish slight changes in

mutants. Therefore, contiguous and wide-ranging changes in inter- and intrapetal variation of pigmentation patterns allow a number of mutants to be distinguished. This may also be the case for flower shape, which is affected by petal shape and number and corolla divergence, and could be different from most other phenotypes, which are recognized only by on-off conversion. Investigation of the expression of transgenes and endogenous anthocyanin biosynthesis-related genes as well as anthocyanin-related pigments might throw some light on this question.

The 53-31 mutant was defective in the expression of the *TjGLO* gene but not in that of the *TjDEF* gene (Figure 3B). This result indicated that such a phenotype (Figure 3Ac) is caused by deficiency in the expression of only *TjGLO* among these two class B genes. Although we expected a specific mutation in the genomic sequence of *TjGLO* which resulted in loss of its expression, we found no such candidate within the sequence that included the 1.4 kb promoter region (Figure 3C). Sequencing analysis of *TjGLO* revealed that its genomic structure, which has five exons, is different from that of the *PI* gene in *Arabidopsis* (six exons; Goto and Meyerowitz 1994) and the *GLO* gene in snapdragon (seven exons; Tröbner et al. 1992). In *Arabidopsis*, the *API*, *LEAFY* (*LFY*) and unusual floral organs (*UFO*) genes have been reported to participate in the expression of class B genes (Weigel and Meyerowitz 1993; Levin and Meyerowitz 1995; Lee et al. 1997; Honma and Goto 2000; Ng and Yanofsky 2001). Since *API*, *LFY*, and *UFO* have yet been cloned in torenia we are now trying to clone these regulatory genes and intend to investigate their expression for future analysis.

Molecular and/or genetic research in torenia is rather difficult because its genomic sequence has not been determined. In this study, however, we isolated many floral phenotype mutants of torenia by ion beam irradiation. For some mutants we were able to identify the deficient gene that causes the mutant phenotype by reference to the abundant information on regulatory genes and/or enzymes in other model plants. We expect that increasing amounts of information on the mechanisms by which floral development is controlled in torenia will accumulate in the future, and this should lead to the production of many useful and desired phenotypes for both scientific and commercial use.

Acknowledgements

We thank Ms. Satoko Ohtawa, Ms. Yoshiko Kashiwagi, and Ms. Yasuko Taniji for maintaining the torenia plants. This work was supported by the Grant-in-Aid "Research Project for Utilizing Advanced Technologies in Agriculture, Forestry and Fisheries" from the Research Council, Ministry of Agriculture, Forestry and Fisheries of Japan (grant no.1783).

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