Mutation Breeding of Thai Native Torenia (*Torenia fournieri* Lind.) by γ-ray irradiation

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Abstract

Gamma ray irradiation is an effective technique to induce mutations for plant breeding. The aim of this research was to develop new cultivars of *Toreni*a (Linderniaceae) for the ornamental market. Axillary buds (1 node cuttings) of new Thai native *Torenia fournieri* Lind. were exposed to acute (Cs-137) gamma ray irradiation at 0, 20, 40, 60, 80 and 100 grays. After transplantation, the survival rate and plant height were measured at 60 d after irradiation. Plant height information was used for calculation of GR_{50}. The results showed that GR_{50} was 68.83 grays. Therefore, axillary buds derived from the first generation of selected mutants were then re-irradiated at 0, 60, 65, and 70 grays. Morphological screening for mutations revealed 3 mutated phenotypes (pink, dark and wavy shaped flowers). The chromosome numbers (2n=2x=18) were not changed after analysis by fluorescence *in situ* hybridization (FISH). The pollen of mutants with wavy flower shape was sterile. The plants with pink, dark and wavy petals were selected for development of new cultivars.

Keywords: Torenia; gamma irradiation; FISH; mutation; axillary bud

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1. Introduction

Torenia sp, a dicotyledon in the family Linderniaceae, is mainly native in Southeast Asia. Many species are found in the tropic, subtropic, humid, rocky and mountainous areas at elevations of 300-1200 m above sea level. Fifty species of *Torenia* were reported including 20 species from Cambodia, Laos, and Vietnam, and 19 species from Thailand (Yamazaki, 1985). Other reports indicate 40 species of *Torenia* (Fischer, 2004; Spencer, 2006). *Torenia* have an extended history of cultivation and as an ornamental plant. *Torenia* have been grown as a front border plant especially in lightly shaded areas and are popularly grown in hanging baskets or as trailing specimens in patio planters (Stamen, 2005). Flowers of *Torenia fournieri* are reportedly edible and can be used as salad material (Shindu *et al*., 2008). Flower color has been modified by genetic transformation by suppressing the dihydro-flovonol-4-reductase (DFR) or the chalcone synthase (CHS) genes that encode enzymes in the biosynthetic pathway of anthocyanins. The anthocyanins are an important pigmemt for flower color (Aida *et al*., 2000)

Torenia sp. is generally a diploid plant in which the basic chromosome number varies in different species. The location and movement of *T. fournieri* chromosomes (2n=18) and centromeres in the early stages of embryo-genesis were observed in interspecific hybrid plants (Kikuchi *et al*., 2005). Tetraploid *Torenia* induced by colchicine treatment and gamma irradiation of young seedlings showed larger flowers than the original diploid flower (Sawangmee *et al*., 2011). This characteristic offers the potential for better development of horticultural varieties. Nevertheless, the pollen viability and seed setting of these tetraploids were significantly decreased when compared with their diploid progenitors due to unequal distribution of chromosomes at anaphase (Tandon *et al*., 1965).

As a different strategy for *Torenia* breeding, Miyazaki (2006) demonstrated that heavy-ion beams can induce flower color mutations at an average mutation rate of 1.06 % (Miyazaki *et al*., 2006). Application of ion beams to *Torenia* sp. also induced mutation to a novel form of flower as reported by Sasaki *et al.* (2008). In addition, γ-rays have been shown to induce flower color mutations and tetraploidy at an average mutation rate of 0.1 % in an interspecific hybrid between *T. fournieri* and *T. baillonii*. However, mutant *Torenia* that exhibit petal color changes may be derived from several gene mutations in the anthocyanin synthesis pathway. Suzuki *et al.* (2000) and Sawangmee *et. al.* (2011) produced yellow flowered plants from transgenic lines of *Torenia* hybrid and γ-ray irradiation in interspecific hybrids between *T. fournieri* and *T. bailonii*, respectively. The yellow colored petals are probably due to the co-suppression of chalcone synthase (CHS) or dihydroflavonol-4-reductase (DFR) genes in anthocyanin synthesis. These yellow-flowered mutants did not demonstrate other changes in phenotype. The research of Boonbongkarn *et al.* (2013) showed that the character of the mutation in mutated Torenia could be maintained by vegetative cutting without reverting back to the original character.

In addition, acute gamma irradiation could induce mutations in flower color and other characteristics in many ornamental plants. Acute gamma irradiation induced mutations have been reported in *Rosa* spp., *Chrysanthemum* spp., *Dianthus* spp., *Dahlia* spp., and *Rhododendron* spp. (Sigurbjornsson and Micke, 1974). Thus, acute irradiation was used to induce mutation in *Torenia fournieri* in the present study.

A new native Torenia found in Thailand (*T. fournieri* Lind.) that has different characteristics from other species was selected for improvement because it is resistant to powdery mildew but is available in only one color. It could be further improved with the introduction of new flower colors, shapes or sizes, which are the most important characters for ornamental plants. Creation of new flowers is an important target for breeding. Thus, we investigated the effects of acute gamma irradiation combined with axillary buds propagation to induce mutation in purple-flowered *T. fournieri* Lind. with the primary focus on the characters of flower color and shape.

2. Materials and Methods

2.1 Plant materials

Purple-flowered native Thai *Torenia* (*Torenia fournieri* Lind.) with a semi-recumbent, semi-erect habit was the subject for this study.

The M_0 (non irradiation) to M_1 (after irradiation in first time) plants were maintained in a greenhouse at a day temperature of 33-35 °C and 60-65 % relative humidity and a night temperature of 29-33 °C and 65-70 % relative humidity.

2.2 γ-ray irradiation

For the acute γ-ray irradiation test, axillary buds (1 node cutting) were irradiated at doses of 0, 20, 40, 60, 80, and 100 grays using a gamma irradiator Mark I machine (J.L. Shepherd & Associates, San Fernando, CA) with a Cs-137 source and then planted in peat moss.

In total, 100 axillary buds per replication (3 replications) were irradiated at each exposure dose. Regenerated shoots appeared by 30 days. The surviving plants and plant height were measured 60 d after irradiation. The GR_{50} was calculated based on the plant height after 60 days.

One thousand axillary buds from the M_1V_2 (after irradiation in first time and propagation second generation) generation were irradiated at doses of 0, 60, 65, and 70 grays using a gamma irradiator Mark I machine (J.L. Shepherd & Associates, San Fernando, CA) with a Cs-137 source and then planted in peat moss.

2.3 Phenotype evaluation and FISH analysis

The plant height, plant spread and flower size of irradiated plants 60 d after exposure were measured with ruler and vernier caliper.

Chromosome preparation from flower buds and the use of FISH for meiotic chromosomes were performed according to the procedures described in Kikuchi *et al*. (2005). A *Torenia* centromeric repetitive DNA sequence (TCEN) was labeled with either biotin-dUTP or digoxigenin-dUTP by PCR or DIG-High Prime (Roche). All images were captured with an Olympus BX61 fluorescence microscope equipped with a cooled-CCD camera (Photometrics CoolSNAP fx: Roper Scientific) and processed using the Meta imaging series 5.0 software (Universal Imaging Corporation) (Jiranapapan *et al.,* 2011).

2.4 Selection of putative mutants

When the plants had grown for 60 days, putative mutants were selected based on morphological characteristics, such as color and shape of flowers.

2.5 Statistical analysis

Statistical differences were tested using Duncan's new multiple range test at the P<0.01 level.

3. Results and Discussion

3.1 Effects of γ-ray irradiation on plant growth

Torenia spp. are known to be easily propagated in tissue culture (Takeuchi *et al*., 1985). In the present study, single nodes were cultured in peat moss and regenerated new shoots from axillary buds. This technique omits several *in vitro* work steps and reduces the regeneration time and acclimatization time required in the production of M_1 plants. After γ-ray irradiation, the regenerated shoots were grown for 60 d, and the mean plant height was recorded as 15.2, 13.8, 10.8, 9.13, 5.73 and 3.86 cm at 60 d for the lateral shoots exposed to 0, 20, 40, 60, 80 and 100 gray of irradiation, respectively. Internode length, crown spread and number of flowers similarly decreased with exposure to increasingly higher rates of gamma irradiation (Table 1). The half growth reduction

Table 1 Plant height, internode length, spread, number of flowers and observed mutations of *Torenia fournieri* Lind. 60 d after gamma irradiation.

Dose	Total plants	Plant height (cm)	Internode length (cm)	Crown spread (cm)	Number of flowers	Number of floral mutants	Mutation rate (%)
0	300	15.2±1.71a	3.00±0.20a	17.83±0.29a	46.33±3.21a	0	0
20	300	13.8±0.92a	2.80±0.20ab	15.50±0.50b	43.33±1.53a	0	0
40	300	10.8±0.60b	2.46±0.06bc	13.83±0.76b	23.67±1.15b	1	0.33
60	300	9.13±0.42b	2.40±0.10c	11.00±1.00c	14.33±4.04c	2	0.67
80	300	5.73±0.61c	2.10±0.15c	7.33±0.58d	0.00±0.00d	0	0
100	300	3.86±0.12c	1.47±0.06d	7.16±1.04d	0.00±0.00d	0	0
F-test	-	**	**	**	**	-	-
% C.V.	-	9.05	5.95	6.15	10.56	-	-

**Means±SD within the same column followed by different superscripts are significantly different using DMRT, $P\leq0.01$; ns = non significant dose (GR_{50}) of acute γ-rays was 68.83 grays (Figure 1). The effect of γ-rays on plant height is dependent on the exposure dose, irrespective of the irradiation method. A similar gradual reduction in plant growth of irradiated plants was noted in interspecific hybrids between *T. fournieri* and *T. bailonii* Sawangmee *et al.* (2011). In the present study, the main morphological change observed in *T. fournieri* Lind. 60 d after exposure to varying doses of acute gamma irradiation compared with the control was small canopy (Figure 2). Apparent mutations in the M_1V_1 plants after acute gamma irradiation were not stable. No solid mutants were obtained after using the cutting back technique (Figure 3). After the GR_{50} was calculated, for the next step of the research 1,000 axillary buds collected from among *T. fournieri* Lind. mutants from the M_1V_2 generation were subjected to irradiation at doses of 0, 60, 65, and 70 grays. The major morphological changes observed in the mutant plants were pink flower color (from γ-ray irradiation at 60 grays) (Figure 4B), dark flower color (from γ-ray irradiation at 65 grays) (Figure 4C) and wavy shaped flower (from γ-ray irradiation at 65 grays) (Figure 4D). Mutant *Torenia* that showed changes in petal color may possibly have resulted from mutation of several genes involved in anthocyanin synthesis. Sawangmee *et al.* (2011) and Suzuki *et al.* (2000) previously reported producing yellow flowered plants from transgenic lines of *Torenia* hybrid and γ-ray irradiation in interspecific hybrids between *T. fournieri* and *T. bailonii*, respectively. The yellow colored petals are probably due to the co-suppression of chalcone synthase (CHS) or dihydroflavonol-4-reductase (DFR) genes in the anthocyanin synthesis pathway. Table 2 shows the plant height, internode length, spread, number of branches, number of flowers, size of flowers, survival rate of pollen and size of pollen 60 d after selected mutant clones from the experiment were irradiated at different

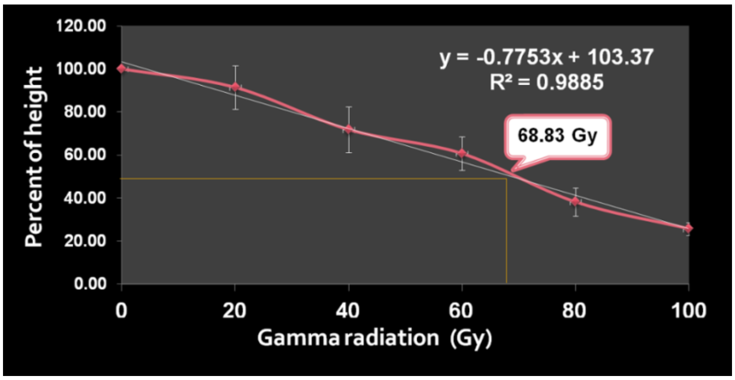

Figure 1 Effect of gamma irradiation (0, 20, 40, 60, 80 and 120 Gy) on plant height (%) of *T. fournieri* Lind. after 60 d exposure. Vertical bars indicated the SD of 300 replications, GR_{50} was 68.83 as shown in figure.

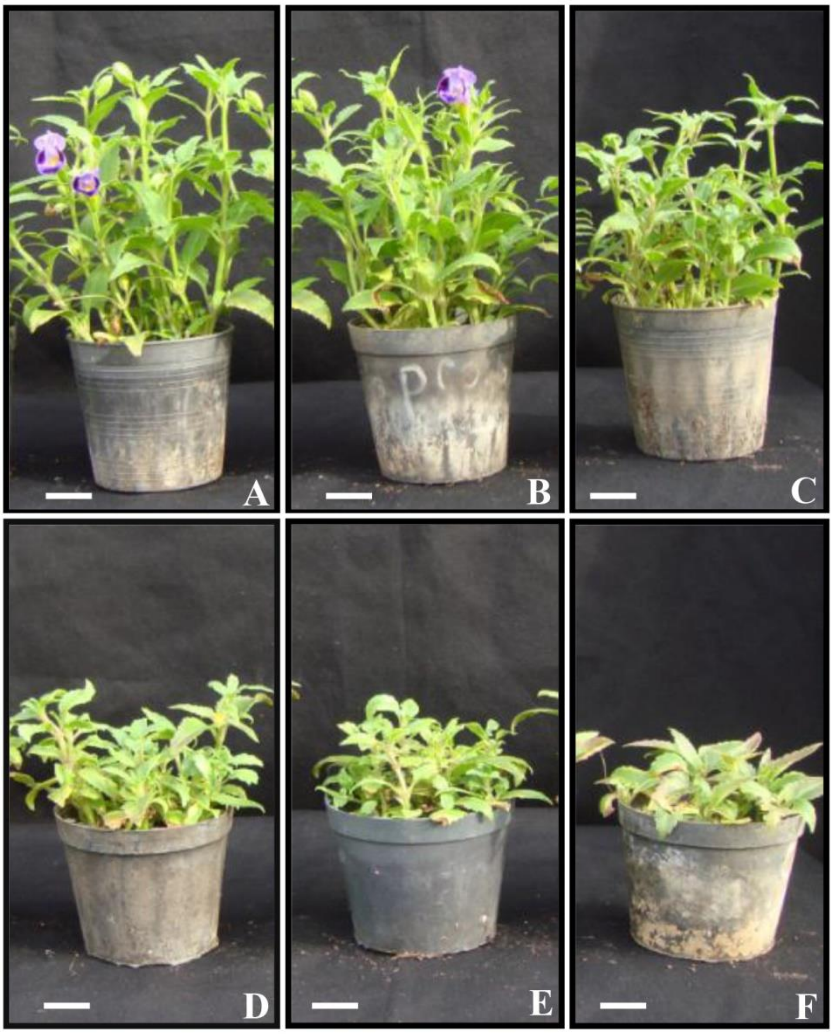

Figure 2 Characteristics of *T. fournieri* Lind. 60 d after exposure to various doses of acute gamma irradiation compared with the control: (A) Wild type; (B) 20 Gy; (C) 40 Gy; (D) 60 Gy; (E) 80 Gy; and (F) 100 Gy. (Bar= 2 cm)

Figure 3 Flowers shape of M_1V_1 generation of *T. fournieri* Lind. after acute gamma irradiation

Figure 4 Flowers from the M_2V_3 generation from re-irradiation of *T. fournieri* Lind. plants propagated by cutting back technique; (A) wild type; (B) pink flower from 60 Gy treatment group; (C) dark flower from 65 Gy treatment group; (D) wavy shaped flower from 65 Gy treatment.

Table 2 Mean plant height, internode length, spread, number of branches, number of flowers, flower size, pollen survival rate and size of pollen of *Torenia fournieri* Lind. 60 d after selected mutant clones were exposed to different doses of gamma irradiation (0, 60, 65, 70 grays)

Plants	Plant height (cm)	Internode length (cm)	Crown spread (cm)	Number of branches	Size of leaves (cm)	Number of flowers	Size of flowers (mm)	Survival of pollen (%)	Size of pollen (µm)
Control	14.33±1.53	2.33±0.29	27.50±4.82	11.33±1.15	2.28±0.29a	42.00±5.29a	23.79±0.40b	8.57±0.91a	32.00±2.00a
Pink	12.67±0.58	1.93±0.12	2.17±2.89	13.00±1.00	1.72±0.22b	35.67±4.04a	17.23±0.21c	81.10±6.52a	23.33±1.15b
Dark	16.33±2.31	2.50±0.00	24.50±1.80	13.67±1.53	1.95±0.11ab	23.00±1.00b	24.52±0.03b	40.57±1.44b	24.00±2.00b
Wavy	13.33±1.15	2.17±0.29	23.00±2.30	10.67±1.53	1.64±0.06b	22.00±2.00b	27.43±0.54a	0.00±0.00c	2.66±1.15c
F-test	ns	ns	ns	ns	**	**	**	**	**
% C.V.	10.78	9.50	13.03	10.87	10.04	11.45	1.52	6.72	7.10

doses (0, 60, 65, 70 grays). Differences in plant height, internode length, crown spread, and number of branches were non-significant, but leaf size was significantly smaller and the number of flowers in some irradiated plants was significantly less than in wild type. The pink color mutant had the smallest flowers and the wavy shaped mutant had sterile pollen (Figure 5D). The color mutants and crown spread observed in the present study were very similar to some reported by Suwanseree *et. al*. (2011), who observed pale blue and pink flower color mutations and some mutants with compact spread after exposing *in vitro* node segments of *Torenia* hybrida to 0-50 Gy of gamma irradiation. Miyazaki *et al*. (2006) observed pale blue, blue, pale pink and bright pink flower color mutations after exposing *in vitro* leaf tissue and internode segments of *Torenia* hybrid cv. 'Summer Wave Blue' to 5-50 Gy of heavy ion beam irradiation. In the present study, stem cuttings were made of mutant plants and the mutations remained stable in 100% of the next generation plants (M_1V_3). Similar morphological changes have been reported in other research; for example, in an induced mutation experiment in carnation, 2 out of 426 lines that were exposed to 50 Gy of heavy ion beam radiation displayed a change in petal shape from serrate to rounded petal (Okamura *et al*., 2003). Similarly, a 2 Gy dose of ion beam radiation resulted in a change in ray floret shape to produce double flowers in 1 out of 1,845 plants of chrysanthemum cultivar H13 when *in vitro* leaves were radiated (Matsumura *et al*., 2010)

3.2 FISH analysis

The chromosome number of 3 types of *Torenia fournieri* Lind. mutants from the irradiation experiments was investigated by FISH analysis to confirm that the morphological differences observed were not due to hybridization with other species. Red signals shown in Figure 6 (A-D) correspond to the TCEN repeat on centromeric region of *T. fournieri* Lind. chromosomes (A-D). The number of chromosomes was not changed from the wild type (2n=2x=18) (Figure 6).

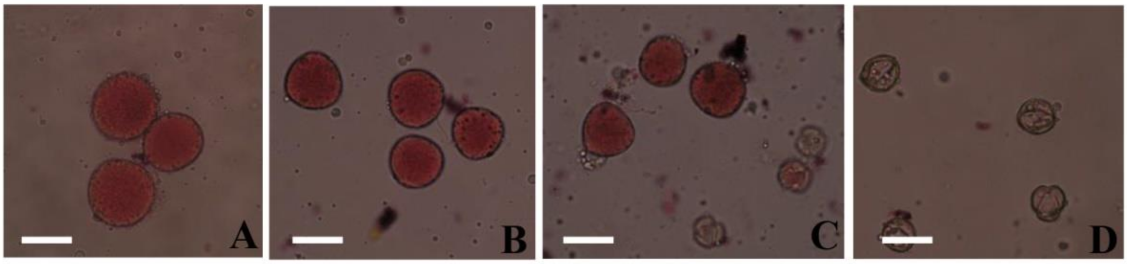

Figure 5 Pollen of *T. fournieri* Lind. mutants after staining with acetocarmine; (A) Wild type; (B) pink flower from 60 Gy treatment; (C) dark flower from 65 Gy treatment; (D) wavy shaped flower from 65 Gy treatment. (Bar = 40 μm)

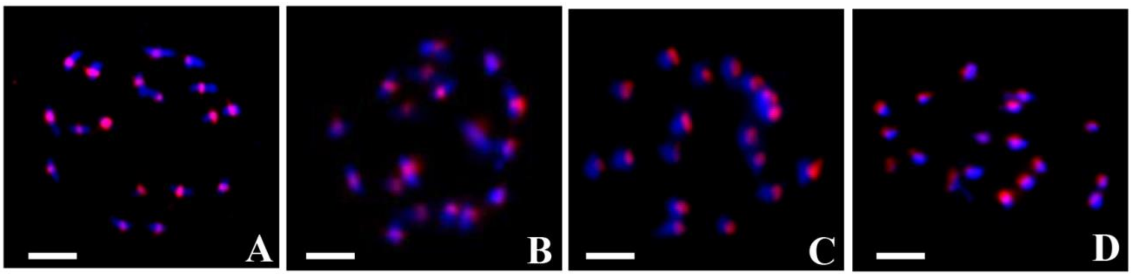

Figure 6 Chromosomes of 4 types of *T. fournieri* Lind by FISH analysis confirming that the mutations are not due to hybridization with other species. Red signals: TCEN repeat on *T. fournieri* Thai (A-D). The numbers of chromosome were not changed. (Bar = 20 μm)

4. Conclusions

Axillary buds of *T. fournieri* Lind. were used as the materials for mutation induction by acute (Cs-137) gamma ray irradiation. After transplantation of gamma treated plants, plant height, internode length, crown spread, number of flowers, number of mutants and flower pattern of original plant and its mutants were observed. The GR_{50} calculated from the plant height after exposure to irradiation was 68.83 grays. The chromosome numbers of the original plant and three mutants were all 2n=2x=18 as observed by FISH analysis.

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