NUCL SCI TECH (2016) 27:112
DOI 10.1007/s41365-016-0117-3

Flower color mutants induced by carbon ion beam irradiation of geranium (*Pelargonium* × *hortorum*, Bailey)

Li-Xia Yu[1,2] · Wen-Jian Li[1] · Yan Du[1] · Gang Chen[3] · Shan-Wei Luo[2] · Rui-Yuan Liu[1,4] · Hui Feng[2] · Li-Bin Zhou[1]

Received: 11 November 2015 / Revised: 20 March 2016 / Accepted: 21 March 2016 / Published online: 26 August 2016

Abstract In an attempt to elucidate the biological effects and underlying mutations involving flower color in ornamental plants following carbon ion beam radiation, shoots of geranium were exposed at dosages of 0, 10, 15, 30, and 40 Gy, and one flower color mutant was obtained. The morphological characteristics, physiological aspects, and DNA polymorphisms between wild-type and flower color mutants were analyzed. The colors of petal, peduncle, pistil, and stamen of the mutant displayed significant differences compared to those of the wild-type. Compared to the original plants, the total anthocyanin content in the petals of the mutant significantly decreased, resulting in a light pink petal phenotype. DNA polymorphisms detected by random amplified polymorphic DNA analysis showed that the ratio of different bands between the wild-type and mutant reached up to 13.2 %. The present study demonstrates that carbon ion beam irradiation is effective in inducing genomic variations, resulting in flower color geranium mutants within a relatively short period of time. Meanwhile, the developed flower-color mutants may be potentially used in future mutational research studies involving ornamental plants.

Keywords Carbon ion beams · Geranium · Flower color mutant · Mutation breeding

Electronic supplementary material The online version of this article (doi:10.1007/s41365-016-0117-3) contains supplementary material, which is available to authorized users.

Li-Xia Yu, Wen-Jian Li, and Yan Du have contributed equally to this research.

This work is supported by the National Natural Science Foundation of China (Grant Nos. 11205218, 11275171, and 11405234), the Knowledge Innovation Project of the Chinese Academy of Sciences (CAS) (No. KJCX2-EW-N05), CAS "Light of West China" Program (No. 29Y506020), and the Youth Innovation Promotion Association of CAS (No. 29Y506030) supported this study.

✉ Li-Bin Zhou
libinzhou@impcas.ac.cn

[1] Biophysics Group, Institute of Modern Physics, Chinese Academy of Sciences, 509 Nanchang Road, Lanzhou 730000, China

[2] University of Chinese Academy of Sciences, Beijing 100049, China

[3] Ningxia Academy of Agriculture and Forestry Sciences, 590 West Huanghe, Yinchuan 750002, China

[4] Gansu Agricultural University, No. 1 Yingmen Village, Anning District, Lanzhou 730070, China

1 Introduction

Cultivated *Pelargonium* spp., also known as 'house geranium' belonging to the family of *Geraniaceae*, is one of the most economical and important bedding and pot plants in North America. These are drought-resistant plants that are widely used as decoration based on their ornamental features. *Pelargonium* has been bred to improve its traits for more than 200 years, and several commercial varieties have been successfully derived from South Africa. There are about 13–17 sections that contain approximately 250 *Pelargonium* species [1]. One of the most popular species, commonly known as a geranium in Europe and North America, is 'zonal' (*Pelargonium* × *hortorum*, Bailey); it is a hybrid and is thought to have originated from *P. inquinans* and *P. zonale*. Another popular cultivated species is 'ivy-leaved', which was obtained from

hybrids of *P. peltatum Horn* subspecies of section Dibrachya.

New varieties of geranium that have been generated by crossing, selection, and spontaneous mutation are generally reproduced by vegetative propagation because their offspring always have decreased fertility. Mutation breeding, especially heavy ion beam radiation-induced breeding, which had been proven to increase natural genetic resources, has successfully assisted in creating new cultivars of seed-propagated or vegetatively propagated plants. These new varieties include *Nicotiana tabacum* that shows tolerance to herbicides, salt, and cobalt or develop altered flowers [2, 3], *Arabidopsis thaliana* that features resistance to ultraviolet-B, anthocyanin spotted testa, serrated petals, abnormal leaves, or premature life cycle [4–7], as well as early maturation, chlorophyll stripped deficiency, wheat mutants with altered plant height, and early maturity sweet sorghum mutants [8–10].

Mutation breeding can also be used in the quality-improvement of ornamental plants. It has been reported that nearly one-fourth of officially released new varieties of mutants around the world are ornamental plants (http://www-mvd.iaea.org). The phenotypes of these new varieties include flowers with varied color, shape, and size, and plantlets with long growth-periods and variegated leaves. The first commercialized variety of ornamental plants obtained using a heavy ion beam mutation breeding technique was the carnation named 'Vital Ion series,' which was reported in 2003 [11]. In addition, the Wandering Jew color-leaf mutant with purple leaves, stems, and petals [12], torenia mutants with varied flower color [13], and *Dahlia pinnata Cav.* dwarf mutants [14] have been developed.

Ion beam irradiation combined with the vegetative propagation technique is an efficient mutation breeding approach for ornamental plants. In 2012, several novel flower color mutants were successfully obtained using this method [15]. Sterile mutants of verbena were isolated from nodal cultures of developed plants irradiated with heavy ion beams [16]. Malformed leaf and variegation mutants of *Saintpaulia ionahta* were induced using carbon ion irradiation combined with plant tissue culture [17, 18].

Flower color is generally caused by three pigments groups, namely flavonoids (the most common one), carotenoids, and betalains [19]. Anthocyanins, a major class of flavonoid molecules, contribute to a wide range of color in flowers. Generally, anthocyanins in plants, including cyanidin, delphinidin, petunidin, peonidin, malvidin, and pelargonidin, are usually linked to one or more sugar molecules through glycosidic bonds. According to published anthocyanin biosynthetic pathways in *Tulipa*, pansy, and petunia [20–24], pelargonidin and cyanidin confer orange to magenta colors to flowers, and delphinidin is responsible for the blue and violet colors of flowers. Early key biosynthetic structural genes such as chalcone isomerase (*CHI*), chalcone synthase (*CHS*), and flavonoid 3-hydroxylase (*F3H*) determine the synthesis of dihydroflavonols. Later, key biosynthesis structural genes such as anthocyanidin synthase (*ANS*) and dihydroflavonol 4-reductase (*DFR*) were determined to be related to the biosynthesis of proanthocyanin and anthocyanin [25, 26].

In 2010, a geranium floral color mutant (*Pelargonium* × *hortorum*, Bailey) was obtained using the combination of carbon ion beam radiation and adventitious shoot regeneration. In the present study, the phenotype, physiological characteristics, and molecular polymorphisms of this mutant were investigated.

2 Experimental section

2.1 Plant material and irradiation conditions

Geranium cultivars (*Pelargonium* × *hortorum*, Bailey) grown under natural light conditions in a greenhouse were used in this study. The buds on young branches (cut and bound together in groups of three or four), were irradiated by vertical 80 MeV/u $^{12}C^{6+}$ beams accelerated by the Heavy Ion Research Facility in Lanzhou (HIRFL) at dosages of 0, 10, 15, 30, and 40 Gy (Fig. 1). The average linear energy transfer (LET) value within each sample as water-equivalent substance was calculated using SRIM2010 program to be 34 keV/μm [27], and the dose rate was 10 Gy/min. A total of 150 excised young branches were treated in this study, namely, 30 branches for each dosage.

Irradiated shoots were transferred into 18-cm flowerpots with regular garden soil and grown under natural

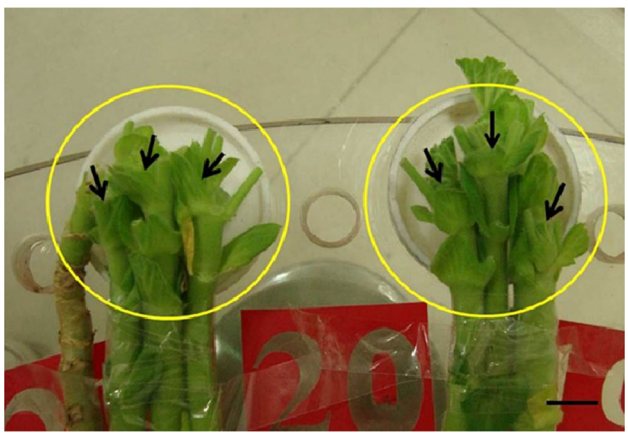

Fig. 1 Geranium plants irradiated by carbon ion beams. *Black arrows* indicate the position of buds in the branches; *yellow circles* show the radiation field of carbon ion beams (Ø 50 mm). *Scale bar* 10 mm. (Color figure online)

temperature and illumination in a greenhouse. Five months later, flower mutants were screened from the treated sample populations of geranium. Since 2010, more than three continuous stem-cutting vegetative propagation culture cycles were performed to maintain the mutagenic phenotype. The stable flower mutant in the third cutting propagation cycle M1V3 was used in the present study.

2.2 Observation of epidermal cells

Isolation of epidermal cells was conducted using the plastic Scotch tape adhesion method. Pieces of 10-plastic Scotch tape (15 cm in length) were laid flat on the laboratory bench with the adhesive side up. Fully expanded petals cut from the plants were carefully stuck to one side of the Scotch tape. The tape on the other side of the petal was pinched and then carefully torn. The tape with samples of epidermal cells was cut into 1 cm × 1 cm square pieces and adhered onto glass slides. The distribution of pigments in the epidermal cells of samples placed on glass slides was examined under a conventional optical microscope at 40× magnification (BX53 Olympus, Tokyo, Japan).

2.3 DNA isolation and polymorphism analysis

Genomic DNA was isolated using a standard procedure [28] with minor modifications. Approximately 3 g of fresh leaves was finely ground in liquid nitrogen, and incubated for 1 h at 65 °C in pre-warmed 2 × cetyl trimethyl ammonium bromide (CTAB) buffer and then washed twice in chloroform:isoamyl alcohol (24:1). After discarding the supernatant, DNA was precipitated with isopropyl alcohol. DNA was rinsed with 75 % ethanol twice, air dried, and dissolved in double distilled water (ddH_2O). Equal quantities of DNA from ten individual M3 plants were pooled for RAPD analysis.

RAPD analysis was performed in a 20-μL mixture that consisted of 20 ng of a DNA template, 10 μM of primers, and a Golden Easy Polymerase Chain Reaction (PCR) System (Tiangen Biotech, Beijing, China), which was mixed with a 2 × reaction mix and 0.25 U of Golden DNA polymerase. A total of 45 cycles of PCR was performed in a programmed temperature control system (Thermal Cycler, BIO RAD, California, USA). The PCR conditions were as follows: First, a 5-min pre-denaturation step at 94 °C; followed by 40 cycles of denaturation at 94 °C for 1 min, annealing at 36 °C for 50 s, and elongation at 72 °C for 1.5 min; and a final DNA synthesis step at 72 °C for 10 min. A total of 55 oligonucleotide primers were used in screening for DNA variations, of which 49 primers (S1) were selected and used in subsequent analyses. Amplified fragments were detected by 1.5 % agarose gel electrophoresis containing Goldview I (Solarbio, Beijing, China) for 1.5 h at 90 V. The amplified DNA fragments were detected and analyzed using a chemiluminescence image analysis system (Tanon, Shanghai, China).

PCR analysis using each primer was repeated three times. All amplified bands, which were stably reproducible, were recorded and described as either absent or present (0/1). The degree of polymorphism between the wild-type and mutant plants was estimated using Jaccard's similarity coefficient, which was determined using the formula equation: $F = 2Nxy/(Nx + Ny)$ [29, 30]; wherein Nxy is the number of identical fragments in two samples; Nx is the number of bands detected in the wild-type; and Ny is the number of bands detected in the mutant.

2.4 Extraction and anthocyanin content assay

Anthocyanins were extracted according to the method of Xie [31] with slight modifications. Immediately after collection, fresh petals (0.5 g) of flowers from the wild type and mutant were ground in liquid nitrogen into a fine powder and suspended in 3 mL of acidic methanol containing 0.1 % HCl. After vigorous vortexing, the samples were placed in the dark for 12 h at 4 °C and shaken every 3 h. The homogenized suspension of petal tissues was rotated in a centrifuge at 2500 g at room temperature for 15 min. The supernatant was collected, and the solid was washed with 0.1 % HCl in methanol. The extraction steps were repeated twice. The supernatants were pooled and the final volume was adjusted to 10 mL in a volumetric flask. Approximately, 2 mL of water and 2 mL of chloroform were mixed with 2 mL of the filtered extract, which was then thoroughly mixed. Next, the mixture was centrifuged at 4000*g* for 10 min. The upper methanol/water phase that contained the anthocyanins was separated, and its absorbance was measured using a UV–Vis spectrophotometer (Agilent 8453, Germany) at a wavelength of 524 nm. Anthocyanin content was expressed as A524 g^{-1} FW.

3 Results and discussions

3.1 Mutation induction in geranium

A flower mutant of geranium was obtained from samples irradiated with 30 Gy carbon ions (Fig. 2). The petals of flowers of this mutant were smaller and thinner than those of the wild type (Fig. 2a) and its color was light pink, whereas that of the wild type was red. In addition, the peduncles of the mutant were red, which significantly differed from that of the wild type. These also had light green pistils and stamens, whereas purple stamens were observed in the mutant (Fig. 2b). The offspring inherited all of these acquired variant phenotypes by cutting vegetative propagation.

Fig. 2 The floral color mutant obtained by carbon ion irradiation in M1V3. **a** Wild-type plant of geranium with *red petals* and mutant with *light pink petals*; **b** The peduncle and floral organ of the mutant are *red* and *purple*, respectively, whereas those of the wild type are *green* and *white*, respectively. (Color figure online)

3.2 Assessment of epidermal cells and distribution of anthocyanins

In plant cells, the distribution and quantity of pigments, particularly anthocyanins, affect flower color [32, 33]. To illustrate pigment distribution in epidermal cells, anatomical observation of petals was performed. Figure 3 shows that the morphology of epidermal cells did not significantly differ between the mutants and the wild type, which were all nearly hexagonal in shape and anthocyanins were distributed in cell vacuoles and intercellular spaces. Orange and red anthocyanins were distributed in the vacuoles of epidermal cells of the wild-type plant, whereas purple and bluish-purple anthocyanins were rarely observed (data not shown). In addition, the color of the lower epidermal cells was lighter than that of the upper cells (Fig. 3a, b). However, the epidermal cells of the mutants were whitish, and colored anthocyanins were hardly observed in the vacuoles (Fig. 3c, d).

No significant differences in the shape of the epidermal cells or the distribution of pigments were observed between the mutant and wild-type plants. Orange and red anthocyanins, which were extensively distributed in the vacuoles of epidermal cells, contributed to the red color of flowers of wild-type plants. In contrast, anthocyanins of lighter color distributed in the vacuoles of the mutant plants rendered a light pink flower color. These results suggested that the difference in petal color between the mutant and wild type was not associated with the shape of epidermal cells, whereas the absence or decrease in anthocyanins in epidermal cells resulted in the light pink color of the flowers of the mutant plants.

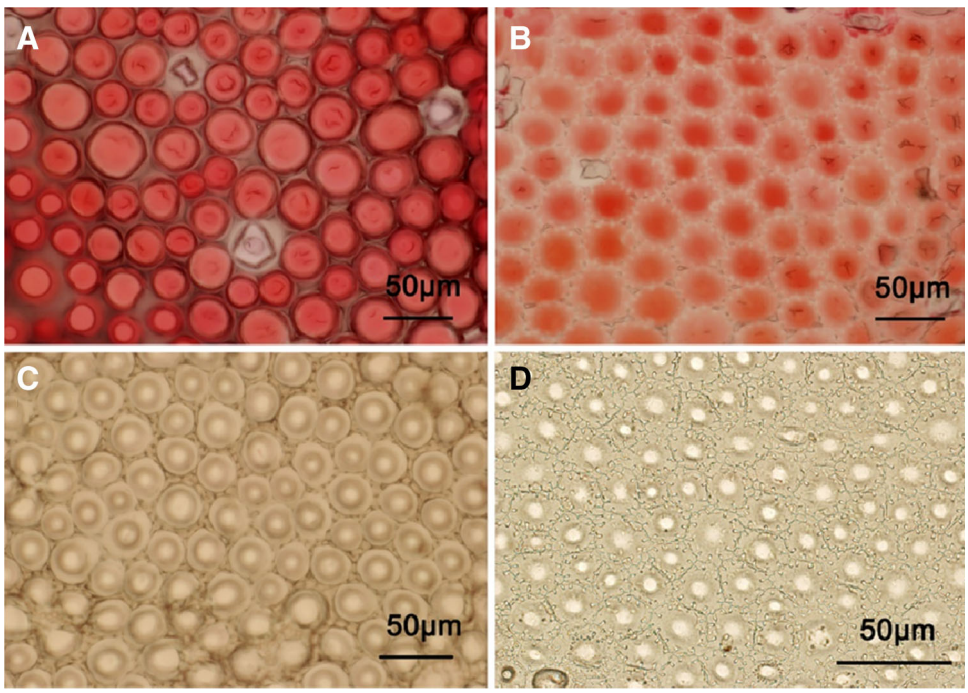

Fig. 3 Microscopic assessment of epidermal cells. **a** The upper epidermal cells of a wild-type plant; **b** the lower epidermal cells of a wild-type plant; **c** the upper epidermal cells of the mutant; **d** the lower epidermal cells of the mutant. *Scale bars* 50 μm

3.3 RAPD analysis of mutants

X-rays, gamma rays, neutrons, and charged particle irradiation can cause DNA strand breaks and clustered lesions [34, 35]. DNA alterations induced by electron and carbon ion irradiations have been reported in *Arabidopsis* [36, 37]. Molecular genetic markers, including those used in RAPD analysis, have been extensively used in detecting DNA alterations [38]. In the present study, differences in DNA fingerprints between the mutant and wild-type geranium plants were characterized using an RAPD assay. A total of 500 recognizable fragments were amplified using 49 different primers. Moreover, the average number of amplified bands per primer set was 10. Among all recognizable fragments, 265 fragments were obtained from the wild type, whereas 235 fragments were amplified from the mutant. A total of 217 fragments was shared by the two fragment populations, thereby indicating that a total of 66 bands were polymorphic. Compared to the wild type, 18 polymorphic bands were identified in the mutant, whereas 48 bands were determined to be residual. The ratio of the polymorphic bands was 3.6 and 9.6 %, respectively. Meanwhile, the similarity coefficient between the mutant and the wild type, which was calculated using Jaccard's formula, was 0.868 (Table S2). Using genomic DNA of the mutant as template, primer P13 amplified a total of 12 bands, whereas only one band was obtained with primer S155. No amplification was observed with primers P12 and P10 in the mutant, whereas 5 and 9 bands were, respectively, amplified in the wild type using the same primers. Figure 4 shows representative polymorphic bands of the RAPD assay involving the wild-type and the mutant flowers.

Goodhead earlier reported that heavy ion beams transferred high energy to the target DNA and induced DNA breaks and large fragment rearrangements such as deletions, translocations, inversions, insertions, and their combinations [39]. These findings indicate that heavy ions could be used as a valuable mutagen for the plant research studies involving forward and reverse genetics. DNA polymorphisms between the control and the early maturity sweet sorghum as induced by carbon ion beam irradiations were successfully investigated using the RAPD technique; and the percentage of polymorphism between the control and the mutant was 5.3 % [40]. Recently, in vitro mutation breeding of *Paphiopedilum* induced by carbon ion beams and gamma ray radiation were undertaken. No variant line was found in gamma ray-irradiated samples, whereas 24 variant lines were screened after carbon ion irradiation. The range of RAPD polymorphisms in the genomic DNA was from 57.8 to 75.6 % between wild-type and variant lines [41]. In the present study, polymorphism fragments of DNA were detected between the wild-type and the light pink flower mutant using the RAPD technique, and the results proved that carbon ion radiation induces genomic variation and DNA polymorphism in geranium. In addition, the present study has shown that RAPD is an effective method for analyzing variations of genome DNA of mutant plants generated by heavy ion beam radiation mutation breeding.

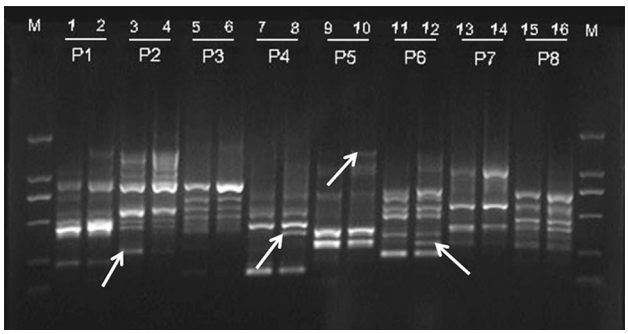

Fig. 4 RAPD analysis of geranium mutants. *M* is the marker; the *odd lanes* are the amplified DNA fragments of the mutant; and the *even-numbered lanes* are those of the wild type. P1–P8 refer to the primers used in amplification. The fragments indicated by *white arrows* are polymorphic bands

3.4 Anthocyanin content analysis

On account of the obvious absence of pink color in the petals of the mutant, the anthocyanin contents of flowers of the mutant and wild type were assessed by UV–Vis spectrophotometry. Figure 5 shows that the amount of anthocyanins that accumulated in the wild type was much higher than that in the light-colored mutant. The anthocyanin content of wild-type flowers was 10 A524 g^{-1} FW, which was about 22-fold higher than that in the mutant, which was only 0.46 A524 g^{-1} FW, and was determined to be statistically significant.

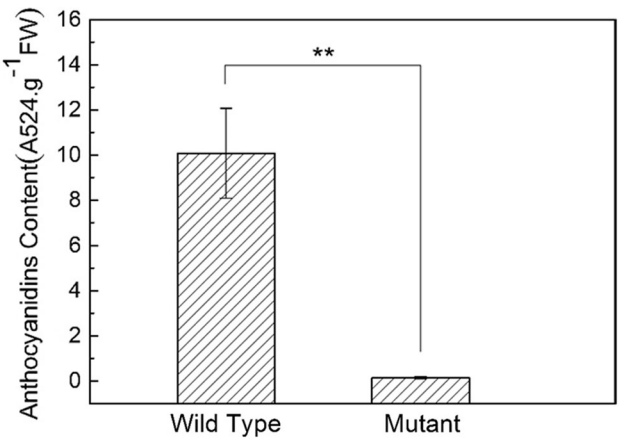

Fig. 5 Comparison of anthocyanin contents of wild type and mutant of geranium flowers. The data represent the averages and standard deviation of three repeat experiments. The student's *t* test was used to determine the statistical significance of the measurements (**$P < 0.01$)

Flower color is generally influenced by the components and quantities of anthocyanins. A previous study has shown that a decrease in anthocyanin content leads to a lighter color [42]. For instance, in gentian, the blue flower of the wild-type plant is associated with high levels of anthocyanins, whereas no detectable pigments were observed in two white-flowered cultivars [43]. Saito showed that in white flower tissues of *Petunia*, a decrease in anthocyanin levels was accompanied by a significant rise in caffeoyl and coumaroyl glucoside concentrations [44, 45]. In the present study, anthocyanin levels in the wild type were significantly higher than that in the light pink-colored mutant, indicating that the marked decrease in anthocyanin content in the mutant might be responsible for the light pink color of the mutant flowers.

Changes in anthocyanin levels are associated with changes in the transcription of key genes that are responsible for anthocyanidin biosynthesis [24]. During anthocyanin biosynthesis, the *CHS* gene is downregulated. Nonetheless, downregulation of the *CHS* gene is not considered as the ideal strategy in developing white-colored varieties. A more feasible preference to induce white-flowered cultivars is to downregulate the *F3H* or *DFR* gene [19]. In spinach and pokeweed, deficiencies in anthocyanin accumulation were attributed to the absence in the expression of the *DFR* and *ANS* genes [46, 47]. Yamamizo determined that the mutations in flower color mutants of *I. quamoclit* and *I. hederifolia* might involve defects in enzymes involved in flavonoid biosynthesis, but not genes that encode transcriptional activation factors. The white flower petals of *I. hederifolia* and *I. quamoclit* were also shown to be deficient in *DFR* and *ANS* expression [48].Two different white-flowered gentian plants were compared to establish the molecular basis of white flower coloration, and the results showed that the white color of Homoi petals was due to a mutation in the structural gene, *ANS*, and the absence of one of two *ANS* loci was confirmed by southern blotting [43]. Taken together, these results suggest that the light pink petal of geranium mutants lack the ability to synthesize anthocyanins, which in turn might be related to the suppression or downregulation of one or more anthocyanin biosynthesis genes. Future studies should thus focus on the transcriptional patterns of anthocyanin biosynthesis genes in this flower color mutant.

In the present study, the color of the peduncles of mutant geranium plants was determined to be red, whereas that of the wild-type was green and colorless. The significance of the red coloration in the stem, peduncle, stamen pistil, and other tissues has been examined. Anthocyanins in the peduncle and stamen provide visual information to pollinators and frugivores [49, 50]. Red pigment in stems indicates a response to abiotic stressors such as drought, low temperature, ultraviolet radiation, or a high ratio of red:far-red light. Cooney earlier showed that peduncle reddening reduces the propensity for photoinhibition, resistance to drought, salinity, heavy metals, or oxidative stress [51]. Previous studies have shown that oxidative stress is one of the major damages induced by heavy ion irradiation; therefore, the red peduncle in mutants might be an adaptive response to heavy ion irradiation.

The spatiotemporal expression of structural genes involved in the biosynthesis of anthocyanins is thought to be determined by regulatory genes and their interactions, which include R2R3-MYB, bHLH, and WD40 factors in *Ipomoea purpurea*, Caryophyllales, and maize [52–54]. In addition, regulatory genes regulate the anthocyanin biosynthesis in various tissues. For instance, in Japanese morning glory, frameshift mutations involving the MYB transcriptional regulator alter the accumulation of pigments in different tissues. The expression of the *InMYB1* gene is only detected in flower tubes and limbs, and the InMYB2 gene is expressed in stems, petioles, and sepals. On the other hand, the mutant with an InMYB1 deficiency exhibits white flowers and red stems [55]. Therefore, variations in transcriptional regulators contribute to color variations in mutants that were generated using heavy ion irradiation. Additional researches on transcriptional regulators in the anthocyanidin biosynthesis pathway and their tissue-specific expression in geranium will be conducted by our group in the near future.

4 Conclusion

In the present study, the shoots of geranium plantlets were irradiated with 80 MeV/u carbon ion beams at dosage of 30 Gy, and one stable mutant with different flower color was obtained. Comparison of phenotypic characteristics, contents and distributions of flower pigments, and DNA polymorphisms between the wild-type and mutant plants showed that carbon ion radiation induces changes in flower color and anthocyanin accumulation in geranium. Therefore, heavy ion beam irradiation may be utilized as a mutagen for flower color improvement and may be proposed as an effective approach for mutation breeding of ornamental plants.

Acknowledgments We express our gratitude to Mr. Huai-An Gu (Institute of Modern Physics, Chinese Academy of Sciences) and Dr. Jinyu He for their invaluable assistance. We also thank our colleagues at HIRFL for providing the carbon ion beams.

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