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Pigment analysis of a color-leaf mutant in Wandering Jew (*Tradescantia fluminensis*) irradiated by carbon ions

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Abstract Many mutants of plant induced by heavy ion beam irradiation have been reported in recent years, but leaf anthocyan mutants induced by ion irradiation in evergreen were rarely found. In this study, a color-leaf mutant with purple leaves, stems and petals was isolated from clones of Wandering Jew irradiated by 95.8 MeV/u carbon ion beam. The concentration and histological distribution of leaf pigment were surveyed in wild type and mutant. In mutant, contents of total chlorophylls (Chl), chlorophyll a (Chl *a*), chlorophyll b (Chl *b*) and carotenoids (Car) decreased significantly, while concentration of the anthocyanins was 6.2-fold higher than that of wild type. Further composition analysis of anthocyanins by electrospray ionization mass spectrometry (ESI-MS) indicated that the purple pigmentation of leaves in mutant was caused by accumulation of petunidin anthocyanin. Microscopic examination showed that most petunidin anthocyanin accumulated in the lower epidermis, and little in vascular parenchyma of mutant, while there was no pigment in wild type. Meanwhile, in spongy parenchyma of mutant we observed little Chl, which the wild type abounds in. In conclusion, the color-leaf mutant of Wandering Jew induced by irradiation of carbon ions was improved in ornamental value, and it could be contribute to variation in level, component and distribution of foliar pigment. The possible mutation mechanisms were discussed.

Key words Foliar pigment, Wandering Jew, Carbon ion beam, Irradiation

1 Introduction

Accelerated ions can induce single- or double-strand DNA breaks with low reparability in bombarded tissue[1], and as an efficient mutagen heavy ion beam irradiation has attracted much interest in horticulture in recent years. Various mutants of ornamental plants have been developed with heavy ion irradiation. Well-known examples include flower-color mutants in carnation[2], rose[3], torenia[4] and chrysanthemum[5], among them the carnation 'Vital Ion series' is the first success in the world as a commercialized new variety by ion irradiation. The involvement of heavy ion beam in flower mutation is well established in various ornamental plants, and there is growing insight into the possible mechanisms. Miyazaki *et al.*[6] obtained pink and blue mutants with color shade variations by nitrogen or neon ion irradiation in *Torenia*, and they attributed the color changes to two additional anthocyanins in flowers of mutants. By carbon ion radiation of *Cyclamen spp*, Kondo *et al.*[7] obtained mutants having red-purple petals**,** while the control had purple flower. They thought that the change in flower color was due to the major anthocyanins transformation from malvidin 3,5- diglucoside to delphinidin 3,5-diglucoside. These results indicated that ion irradiation is an effective method to create novel flower color, which is highly dependent on concentration, category and distribution of anthocyanins.

Leaf mutations induced by ion beams were reported less frequently, and most of the authors focused on abnormal development of leaves. Wu *et al.*[8] found bileaf, trileaf and tetraleaf conglutination in

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Salvia splendens Ker-Gawl irradiated by carbon ions and the mutants were inhibited in all growth stages without any influence on blossom- and seed-bearing. Narrow and spotted leaf mutants were also observed in *Oncidium lanceanum* treated by carbon ion beam[9]. Ion beam-induced leaf mutation of ornamental plant has its potential in horticulture, however, it is very rare to find irradiated evergreen mutant with leaves deficient in chlorophylls and rich in anthocyanins.

Wandering Jew is a perennial evergreen herb of *Tradescantia.* Plants of this genus are often used as indoor or outdoor ornamental plant due to their trailing ground covering succulent stems, and to their easy growth and propagation[10], but they are high sensitive to heavy metal pollutants and ionizing radiations. *Tradescantia* micronucleus and stamen hair mutation assays are used to test genotoxicity of environmental factors[11]. We presumed that Wandering Jew might be efficient to be mutated by heavy ion irradiation.

By carbon ion irradiation of Wandering Jew, a mutant with purple leaves, stems and flowers was obtained. Study on the color-leaf mutant might throw some light on effects of the ion beam irradiation on foliar pigment variation and leaf development. In this paper, we characterize the color-leaf mutant of Wandering Jew induced by carbon ion irradiation, and analyze its foliar pigment and discuss possible mechanisms.

2 Materials and Methods

2.1 Plant material and irradiation

The carbon ion beams were from the Heavy Ion Research Facility in Lanzhou (HIRFL), with average energy of 95.8 MeV/u. Five shoots (10–12 cm length) of Wandering Jew picked from greenhouse-grown plants were tied up and irradiated at the growing points to different doses of up to 20 Gy at dose rate of 4 Gy/min. Each irradiation treatment was performed in triplicate.

After irradiation, the shoots were transferred by stem cutting into Φ18 cm flowerpots with regular garden soil as a substrate, and grown in a greenhouse under natural conditions of light and temperature. About 7 months later, mutant of Wandering Jew with color leaves was screened (the 20-Gy treated), and the mutated plant have been maintained and propagated by stem cutting since 2007. All materials in this paper were collected in mid November, when pink areas appeared apparently in the first three leaves of mutant. The anthocyanins were tracked every two months.

2.2 Assessment of chlorophylls and carotenoids

Chlorophylls and carotenoids in fresh leaves were extracted with 80% acetone for spectrophotometry analysis. The concentrations of total chlorophylls (Chl), chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b)*, and carotenoids (Car) were calculated using the equations of Lichtenthaler and Wellburn[12].

2.3 Extraction and estimation of anthocyanins

Anthocyanins were extracted following the method of Xie *et al.*[13] with slight modification. Briefly, 1 g of fresh leaves was ground to fine powder in liquid nitrogen, and suspended in 5 mL acid methanol containing 0.1% HCl in 10 mL screw-cap polypropylene tubes. After vigorous vortexing for 30 s, the tubes were shaken for 1 h at 120 rpm, and centrifuged for 15 min at 2500 g at approximately 20ºC. The extraction step was repeated twice, and supernatants were pooled and adjusted to 25 mL volumetric flask.

The extract of 1 mL was mixed with 1 mL water and 1 mL chloroform. After vigorous vortexing for 10 s, the tubes were centrifuged for 15 min at 2500 g at 20ºC. The chloroform phase containing chlorophyll was separated from the methanol/water phase containing anthocyanins. Absorbances of the upper methanol/water phase were measured at 524 nm with an UV-Vis spectrophotometer (Agilent 8453, Germany). The anthocyanins content was expressed as $A524 \cdot g^{-1}$ FW.

2.4 Electrospray ionization mass spectrometry (ESI-MS) analysis of anthocyanins

Anthocyanins extract was filtered through a 0.45 μm filter paper after condensed in a rotary vacuum evaporator at 40ºC. Further analysis of anthocyanins was performed by low-resolution electrospray mass spectrometry with an Esquire 6000 ion trap mass spectrometer (Bruker Daltonics, Billerica, MA). Positive ionization mass spectrometry was processed, scanning from *m*/*z* 200 to 1500. Identification of

individual anthocyanins was performed based on molecular weight and published data.

2.5 Histological localization of anthocyanins

Fresh leaves were collected at the same position, and purple region of the mutant leaf lamina was preferentially selected. Transverse sections were made by hand sectioning, and the lower epidermises were peeled from leaf tissues using tip forceps. The samples were observed under an optical microscope (XSP-8CA, Shanghai Optical Instrument Factory), and images were taken with a digital camera (DSC-W50, Sony).

2.6 Statistical analysis

Data from independent experiments in triplicate were analyzed by variance analysis (ANOVA), significant difference between wild type and mutant was obtained by Student's t-test using SPSS 16.0 software. The data were expressed in means ± standard deviation. The data marked with * are of significant difference ($p<0.05$), and data marked with ** are of highly significant difference ($p<0.01$), between wild type and mutant of Wandering Jew.

3 Results

3.1 Phenotypic characteristics of color-leaf mutant and wild type

Observations on the color-leaf mutant of Wandering Jew indicated that light pink patches appeared on the basal part of adaxial surface of the young leaves in the first two weeks of September, with purple abaxial surface. Pink patch enlarged gradually, and it did not fade until March of the next year. The pigment mutation has been maintained by consecutively vegetative reproduction. A1and A2 in Fig.1 shows the plants of wild type and mutant in mid November. More detailed changes of the mutant are shown in B1–B8 of Fig.1. The mutated leaf was a variegated chimera (Fig.1, B2 and B4). The superficial pigmentation pattern and proportion of pink and green/purple marking on leaf lamina were obvious. At proximal end of petiole, the adaxial surface of leaf blade was light pink, and the corresponding area of abaxial side was purple. Meanwhile, the adaxial and abaxial sides at distal end of petiole were dark green and mosaics of green and purple, respectively. Moreover**,** purple coloration also appeared on stem, petals and flower stalk of mutant, and chlorophylls-deficient mutation was observed on calyces of mutant in Wandering Jew. However, leaf, stem, flower stalk and calyces of wild type remain green all the year, except that petals were white.

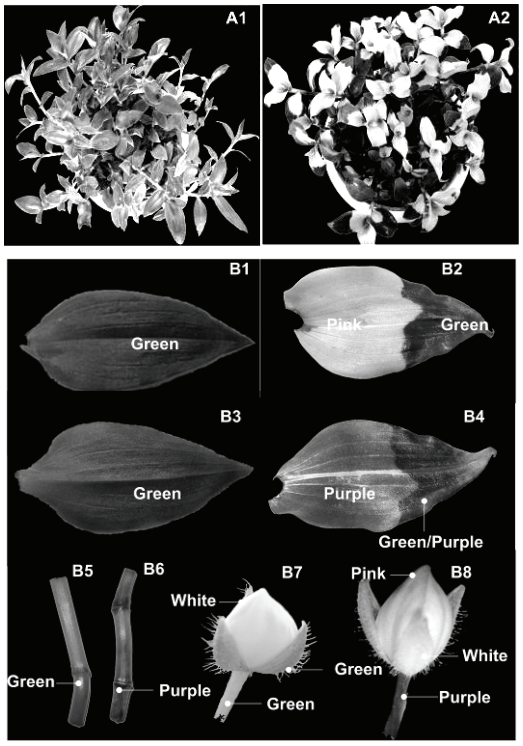

Fig.1 Phenotypic comparison of wild type and mutant of Wandering Jew. (A1) wild type with evergreen leaves; (A2) mutant with color leaves. (B1) adaxial and (B3) abaxial surfaces of the leaf blade of wild type were green; (B2) adaxial and (B4) abaxial surfaces of the leaf blade of mutant presented pink and purple; stems of wild type (B5) and mutant (B6) were green and purple respectively; (B7) white petals of wild type was with green flower stalk and calyces; (B8) light pink flower of mutant with purple flower stalk and chlorophyll-deficient calyces.

3.2 Pigment analysis and determination of color-leaf mutant and wild type

Leaf color is inevitably related to pigment such as chlorophylls, carotenoids and anthocyanins. Among them, anthocyanins are associated with pink to purple pigmentation in leaves and other organs[14]. Since the evident purple pigmentation and variation on leaves of mutant, anthocyanins levels were followed every other month in a year. In Fig.2, the levels of anthocyanin in wild type were very low and remained more or less constant, while mutant with anthocyanin was much higher. It has a curved variation during the year, being

the highest in January, the lowest in July with the content being almost the same as that of wild type, and the highest again in November.

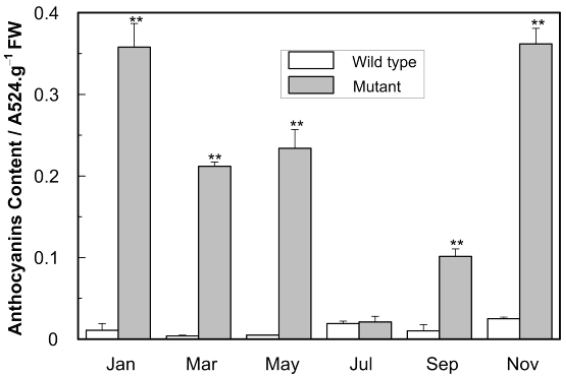

Fig.2 Variation of foliar anthocyanins of wild type and mutant of Wandering Jew during a year. Highly significant differences were found between wild type and mutant in all periods except in July.

The foliar pigment of mutant and wild type grown in November was studied. As shown in Fig.3, the Chl, Chl *a*, Chl *b* and Car concentrations declined significantly, while anthocyanins were overproduced in leaves of the mutant, with the mutant anthocyanin being 8.21-fold higher than that of the wild type.

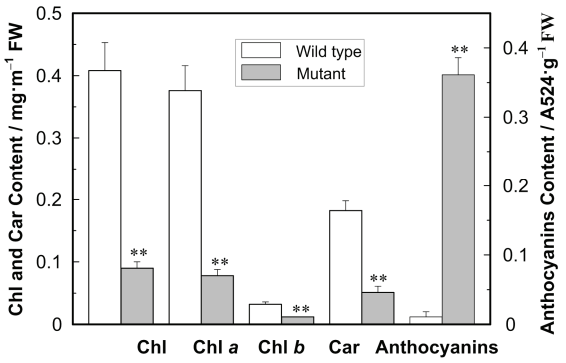

Fig.3 Foliar pigment of wild type and mutant of Wandering Jew. Highly significant differences were found between wild type and mutant in all types of pigment detected.

3.3 Anthocyanins identification of color-leaf mutant and wild type

Anthocyanins are a group of widespread natural pigment in plants, composed of an aglycone anthocyanidin and some sugar moieties. Common anthocyanidins found in plants include cyanidin, delphinidin, petunidin, peonidin, malvidin and pelargonidin. And one or more sugar molecules can be linked to anthocyanidin through a glycosidic bond. It is reported that there are over 600 natural anthocyanins, and all are O-glycosylated with different sugar substitutes and acylated groups[15]. Plant anthocyanins identification can be done with MS technologies of electrospray ionization mass spectrometer (ESI-MS), electron impact mass spectrometer (EI-MS), atmospheric pressure chemical ionization mass spectrometer (APCI-MS), and matrix-assisted laser desorption/ ionization mass spectrometer (MALDI- MS).

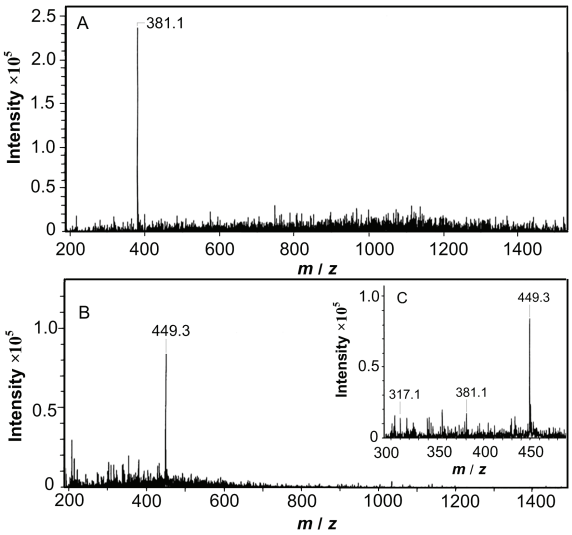

Fig.4 ESI-MS spectra of anthocyanins of wild type and mutant. (A) wild type, (B) mutant, and (C) an enlarged section of ESI-MS spectra of mutant scanning 300–500 nm.

The ESI-MS was used to analyze anthocyanins in the wild type and mutant of Wandering Jew. Individual anthocyanins were identified by comparing their mass spectral data with published data. As shown in Fig.4, distinctive peaks with the highest intensity appeared on either ESI-MS spectra A and B, which suggest different components in extract of wild type and mutant. In Fig.4B, the peak of the highest intensity in leaf extract of mutant has a parent cation of *m/z* 449 and a fragment of *m*/*z* 317, and it was tentatively identified as a pentoside of petunidin or petunidin 3-arabinoside. Yet no anthocyanins were evident in wild type (Fig.4A). The most abundant peak with *m*/*z* 381 appeared in ESI-MS spectra of wild type, while it was found in much lower intensity with the mutant. The peaks of much lower intensity in the MS spectra of both wild type and mutant might be resulted from undesirable products such as sugars, acids, amino acids and proteins that could interfere with analysis of anthocyanins.

3.4 Cellular localization of pigment in color-leaf mutant and wild type

Microscopic examination indicated that in variegated leaf of mutant, anthocyanins were evident as purple solution inside cell vacuoles of the lower epidermis and vascular parenchyma as discrete cell clusters or as individual cells, and the spongy parenchyma held an unidentified brown or black pigment in most cells (Fig.5B). In the lower epidermis of mutant leaf, anthocyanins were prone to locate next to stomata (Fig.5D). In leaf of wild type, no pigment was observed in any of these cell types, except that the spongy parenchyma was filled with green coloration intensely that could be ascribed to chlorophylls mainly (Fig.5A).

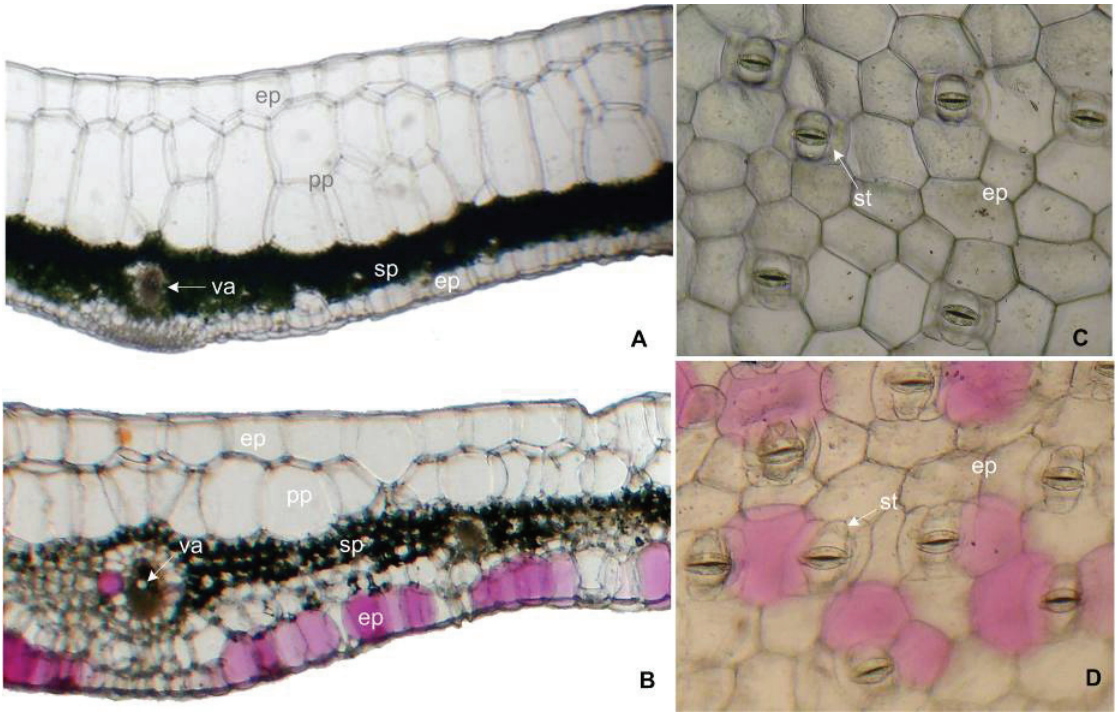

Fig.5 Light microphotographs of transverse sections through leaf midribs of wild type and mutant of Wandering Jew. (A) Green leaf of wild type with abundant chlorophylls in spongy parenchyma; (B) pink leaf of mutant with anthocyanins in some cells of lower epidermis and vascular parenchyma; (C) no pigment in the foliar lower epidermis of wild type; (D) anthocyanins presented in the foliar lower epidermis of mutant. ep, epidermis; pp, palisade parenchyma; sp, spongy parenchyma; va, vascular bundle; st, stoma.

4 Discussion

Leaf color is of important commercial value for an ornamental foliage plant. To our knowledge, available information about mutant that has anthocyanic leaf originated from evergreen induced by heavy ions is scanty. Here, distinct characteristics of color-leaf mutant of Wandering Jew induced by carbon ions are described, and foliar pigment are analyzed. Chlorophylls and carotenoids, as the main pigment in green leaves, were decreased drastically in variegated leaves. And the overproduced anthocyanin in the mutant was tentatively identified as petunin anthcoyanin, which was restricted to some cells of lower epidermis and vascular parenchyma.

There are three types of pigment (chlorophyll, carotenoids and anthocyanins) in plant leaves, and their content or distribution determines the color of leaves. Usually, high concentration of chlorophyll predominates in green leave plants. And chlorophyll deficiency in leaf is a well known mutation induced by heavy ions. Abe *et al.*[16] screened rice mutants with albino leaf in M2 progeny with the seeds irradiated by carbon and neon ions, respectively. The mutation was attributed to deletion of a DNA fragment. In estimating the mutation rate of *Arabidopsis thaliana* induced by carbon ions, mutants without pigment in leaves were isolated at the M2 generation[17]. Histogenic studies on chlorophyll-decreased leaf of tobacco were conducted by Bae *et al.*[18], who discovered green-white variegations generated in leaves after heavy ion irradiation, and explained the

phenomenon by an absence of developed chloroplasts in the epidermis and palisade parenchyma. In present study, decreased chlorophylls in color-leaf mutant of Wandering Jew were detected. No green pigment was observed in spongy parenchyma of mutant, while the tissue of wild type is green. For variegated plants, cells in the white sectors appear to be blocked at various stages of chloroplast biogenesis and they can be caused by mutations in nuclear, chloroplast or mitochondrial genes[19]. Therefore, the unidentified coloration in spongy parenchyma of mutant might be involved in abnormal palstids that lack chlorophylls and carotenoids.

Anthocyanins are possibly ubiquitous in green leaves at low quantities[20], which are often synthesized under stress conditions, such as high-light, cold temperature, nutrient deficiency or pathogen attack[21]. As mentioned in introduction, variations of anthocyanins influenced by heavy ions were reported in flowers mostly, fewer were found on leaves. Rice mutant line BKOS6 with red/purple color in leaf sheath, collar, auricles, ligule, and dark brown stripes on leaf blade, were selected by Phanchaisri *et al.*[22], with seeds irradiated by nitrogen ions. Analysis of genomic variation suggested two additional DNA bands at 450 bp and 400 bp. However, variations in content and component of anthocyanins in rice mutant were not mentioned. In our research, foliar anthocyanins in mutant of Wandering Jew were recorded in a year. Although purple color existed in mutant leaf all year round, the concentrations varied in a season-dependent manner (Fig.2). This could be attributed to environmental conditions such as temperature and light, which were essential factors for stability of anthocyanins in plants[23]. Furthermore, petunidin was identified as the additional anthocyanins in leaves of mutant. From the known biosynthetic pathway of anthocyanins, the accumulation of petunidin in mutant indicates that the expression of related structrual or/and regulatory genes may be enhanced in color-leaf mutant.

5 Conclusions

A color-leaf mutant with remarkable changes in Wandering Jew was induced by carbon ion beam irradiation. Since the apparent appearances of different cell layers, it was confirmed that pigment patches on variegated leaf were formed due to the complementation of petunidin anthocyanins in lower epidermis and black/brown or green coloration in spongy parenchyma. Variegated plants could be non-inheritable or inheritable. Inheritable ones are generated by lesions in nuclear, plastid or mitochondrial genes, and the mutated characteristics can be transmitted to progeny cells. However, some non-inheritable mutations can also survive over many generations in vegetatively propagated plants[24]. Although the phenotypic change in mutant of Wandering Jew induced by carbon ions radiation was stable in vegetative propagation, it was difficult to deduce the nature of mutation. A detailed molecular analysis to identify the genetic alterations is needed.

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