Analysis of bioactive compounds in gamma irradiation-induced citrus mutants

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Abstract: This work aimed to evaluate the effect of gamma irradiation on phytochemical components such as phenolics in citrus fruit. A branch of citrus derived from 120 Gy gamma irradiation produced fruit peel and pulp with high contents of total phenolics, total flavonoids and pigments. Moreover, all flavonoids (naringin, hesperidine, narirutin and rutin) detected by UPLC analysis demonstrated significant alterations in citrus mutants. These findings suggest that gamma irradiation on citrus may produced mutants enriched in bioactive phenolic compounds.
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1. Introduction

For more than a century, geneticists and citrus breeders have pursed various developmental experiments on new citrus cultivars. With biotechnology entering the commercial crop breeding industry, citrus breeding programs are now found in many parts of the world. Improvements in citrus fruit productivity, pest control, disease resistance, tolerance to environmental stress, and characteristics of fruit quality are expected to result in greater economic gain to citrus fruit cultivators (Sarto et al., 2006; Naito et al., 2005; Predieri, 2001; Ahloowalia and Maluszynski, 2001).

Bioactive compounds in citrus fruits have been shown to be protective against chronic diseases such as cancer and heart disease (Molina et al., 2010). Our research group has reported the phenolic composition of gamma irradiation-induced citrus leaves and fruits (Kim et al., 2012). Nevertheless, little research has been done in the influence of gamma irradiation on bioactive component of citrus, and particularly on citrus fruit prepared by gamma irradiation of budsticks nothing has been reported. Herein, we describe the alterations of various bioactive compounds in citrus mutants obtained by gamma irradiation.

2. Material and Methods

2.1. Gamma irradiation

One year old shoots of citrus, *Citrus unshiu* Marc. cv. Miyagawa, was irradiated with gamma ray (120 Gy) to induce the formation of mutants and maintained as described previously (Kim et al., 2012).

2.2. Preparation of methanolic extracts of citrus WT and Mut

Both citrus wild-type (WT) and mutants (Mut) fruits obtained from same citrus tree were harvested in November, 2011, and grouped by morphological characteristics as follows: Mut I, citrus mutants with red color; Mut II, citrus mutants with the large sized, rough peel shape and comparatively high sugar/acid ratio; Mut III, citrus mutants with the pinnate leaves, large sized, rough peel shape and comparatively low sugar/acid ratio; Mut IV, citrus mutants with rough peel shape. Each WT, citrus derived from non-irradiated shoots, was correspond to each Mut.

Citrus fruits were washed by distilled water then peeled and their edible portions were carefully separated. They were lyophilized, ground to a fine powder and passed through a mesh sieve. 25 g powdered sample was extracted with 500 mL of methanol at the 25 ℃. The mixture filtered through a Whatman No. 2 filter paper for removal of particles. The residue was re-extracted twice under the same condition to ensure complete extraction. The extracts were placed in dark bottles and stored in refrigerator at 4 ℃ until use.

2.3. Analysis of total phenolic and flavonoid contents

Contents of total phenols and flavonoids were determined by the method described previously (Kim et al., 2012).

2.4. UPLC-MS analysis of flavonoids

UPLC was performed on an Acquity Waters Ultra Performance Liquid Chromatographic system equipped with an Acquity photodiode array (PDA) detector coupled with a triple quadrupole tandem mass spectrometer (Micromass® Quattro microTM API, Waters, Milford, MA, US) with electrospray ionization (ESI). Flavonoids (naringin, hesperidin, hesperetin, narirutin, rutin) were separated on a UPLC BEH C18 column (50 mm x 2.1 mm, 1.7 μm particle size). The column temperature was kept at 33 °C. Optimum separation was achieved with a

binary gradient of 0.1% formic acid (vol/vol) in water (solvent A) and acetonitrile (solvent B) at a flow rate of 0.2 mL/min under the following program: 0–0.63 min, 2% B; 0.63–3.50 min, 2–60% B; 3.50–4.00 min, 60% B; 4.00–4.50 min, 60–2% B; 4.50–5.00 min, 2% B (equilibration of column). The injected sample volume was 2 µL and the UV spectra by PDA were recorded between 210 and 410 nm.

MS detection was performed directly after PDA measurements. The ESI source was optimized with positive ionization mode as follows: scan spectra from *m/z* 100 to 400, capillary voltage 3.30 kV, cone voltage 20 V, source temperature 120 °C and desolvation temperature 360 °C. Nitrogen was used as the desolvation and cone gas with a flow rate of 850 and 50 L/h, respectively. For quantitative determination of the six flavonoids, the MS detection was operated in negative ESI mode with multiple reaction monitoring (MRM). Argon was used as the collision gas at a pressure of 3.82×10^{-3} mbar. Instrument control, data acquisition, and quantification were performed by Mass Lynx 4.1 software (Waters).

2.5. Analysis of chlorophylls and total carotenoid

An aliquot of the methanolic solutions of citrus peel and pulp extracts was used for quantification of the contents of chlorophylls a and b, and total carotenoids using a Spectra MR microplate reader (Dynex Technologies, Inc., Chantilly, VA, US). The chlorophylls a and b, and total carotenoid contents were calculated by measuring the absorbance at 470, 653 and 666 nm according to the equations reported previously (Rainha et al., 2011). All operations were carried out on ice under dim light to prevent photodegradation, isomerizations, and structural changes of the carotenoids.

2.6. Analysis of β-carotene and lycopene

For β-carotene and lycopene determination a fine dried powder (15 mg) was vigorously shaken with 1 mL of acetone–hexane mixture (4:6) for 1 min in the dark room. The absorbance of the filtrate was measured at 453, 505, 645 and 663 nm and calculated according to the Guimaraes et al. equations (2010).

2.7. Statistical analysis

Comparisons of all results between the Mut and WT samples were made by using a nonparametric test (Mann-Whitney U test) with $P < 0.05$ (SPSS, ver. 12.0; SPSS Inc., Chicago, IL, US). For each measurement, three replicate samples were tested.

3. Results and Discussion

Total phenolic and flavonoid contents of citrus peel and pulp extracts are shown in Figure 1. A similar quantity was observed in the total phenolic content of the citrus Mut peel and pulp extracts compared to the wild-type (Figure 1A). However, the flavonoid contents of citrus Mut peel and pulp were significantly higher than those of WT; a noticeable increase in total flavonoid content of Mut III (Figure 1B).

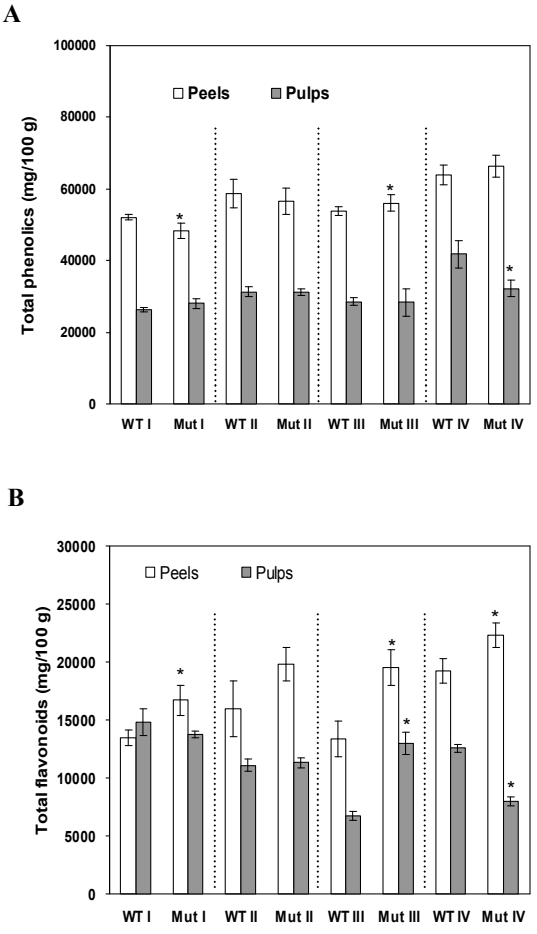

Fig. 1. Total phenolic (A) and flavonoid (B) contents in the peel and pulp extracts of citrus derived from non-irradiated and irradiated shoots. Data presented are in mean ± standard deviation ($n = 3$) which with asterisk (*) above the bars are significantly different at $p < 0.05$.

Table 1. Flavonoid content (mg/100 g dry weight) in the peel and pulp extracts of citrus derived from non-irradiated and irradiated shoots

Group	Flavanone				Flavonol glyside
	Naringin	Hesperidin	Hesperetin	Narirutin	Rutin
Peel					
WT I	12.3 ± 0.42	79.9 ± 0.11	nd†	13.1 ± 0.25	1.3 ± 0.03
Mut I	17.1 ± 0.03*	44.6 ± 1.08*	nd	17.4 ± 0.21*	0.6 ± 0.08*
WT II	12.5 ± 0.36	77.1 ± 1.21	nd	11.5 ± 0.20	1.3 ± 0.08
Mut II	15.6 ± 0.16*	90.6 ± 1.00*	nd	16.2 ± 0.11*	1.5 ± 0.04*
WT III	12.5 ± 0.12	73.8 ± 5.29	nd	10.8 ± 0.57	1.6 ± 0.16
Mut III	21.4 ± 0.04*	59.0 ± 0.90*	nd	21.2 ± 0.18*	1.1 ± 0.00*
WT IV	12.6 ± 0.08	75.3 ± 0.85	nd	12.2 ± 1.34	1.3 ± 0.04
Mut IV	22.3 ± 0.21*	125.6 ± 1.01*	nd	22.4 ± 0.29*	1.5 ± 0.03*
Pulp					
WT I	21.2 ± 0.23	307.8 ± 5.17	nd	21.6 ± 0.40	8.1 ± 0.03
Mut I	30.7 ± 0.37*	302.3 ± 3.93	nd	30.7 ± 0.30	1.6 ± 0.29*
WT II	21.2 ± 0.04	307.1 ± 1.21	nd	21.5 ± 0.20	7.6 ± 0.50
Mut II	34.5 ± 0.23*	398.4 ± 4.18	nd	34.5 ± 0.64	10.5 ± 0.18*
WT III	21.4 ± 0.01	307.1 ± 5.18	nd	21.2 ± 1.09	7.6 ± 0.16
Mut III	41.2 ± 0.23*	308.8 ± 1.40	nd	42.1 ± 0.13	5.6 ± 0.01*
WT IV	21.5 ± 0.01	305.3 ± 0.85	nd	22.2 ± 1.34	7.9 ± 0.45
Mut IV	43.3 ± 0.45*	431.6 ± 2.37	nd	43.7 ± 0.82	13.2 ± 0.13*

† Not detected; * Values are significantly different from corresponding WTs ($p < 0.05$).

Citrus fruits and juices are an important source of bioactive fruits and juices are an important source of bioactive compounds including antioxidants such as ascorbic acid, flavonoids, phenolic compounds and pectins that are important to human nutrition (Ebrahimzadeh et al., 2008; Fernandez et al., 2005; Jayaprakasha et al., 2008). Flavanones, flavones and flavonols are three types of flavonoids that occur in citrus fruit (Calabro et al., 2004). The main flavonoids found in citrus species are hesperidine, narirutine, naringin and eriocitrin (Schieber et al., 2001). Epidemiological studies on dietary citrus flavonoids reduce the risk of coronary heart disease (Hertog et al., 1993; Majo et al., 2005). Also, it was attracting more attention as anti-carcinogenic and anti-inflammatory agents because of their lipid anti-peroxidation effects (Tripoli et al., 2007; Hegazy and Ibrahium, 2012).

Each pair of WT and Mut citrus fruits studied in this paper were collected in the same tree and date, therefore, were produced under the same conditions to reduce this additional source of variance. Table 1 summarizes the quantitative determination of four flavanones (naringin, hesperidin, hesperetin and narirutin) and one flavonol (rutin) in citrus peel and pulp extracts. Citrus fruits pulp contained higher level of investigated flavanones and Flavonol glyside than peel. Hesperidin was the most abundant flavonoid in the citrus samples studied, followed by naringin (or narirutin) and rutin. No hesperetin was found in the samples. The amounts of naringin, hesperidin, narirutin and rutin were significantly higher in peel and pulp extracts of all Mut groups than WT ($p < 0.05$), except for rutin of Muts I and III peel and pulp extracts (Table 1).

Table 2 presents chlorophyll a, chlorophyll b, total carotenoid and lycopene concentration obtained in the citrus fruits studied. Carotenoid was the major components found in the extracts (56-240 μg/g). β-carotene and lycopene were found in small amounts (0.5-3.5 μg/g), and chlorophyll a and b were found in only vestigial amounts (<0.09 μg/g). Carotenoids are important to humans because of their nutraceutic property. The carotenoid lycopene and β-carotene pigment are responsible for the red and yellow color of the fruit, respectively.

Carotenoids in citrus fruits are localized in plastids present in both the flavedo and in the vesicles that contain the juice. When the fruit is immature their color is masked by chlorophylls, with the progress of ripening yellow appears in various shades

Table 2. Color pigment content (μg/g dry weight) in the peel and pulp extracts of citrus derived from non-irradiated and irradiated shoots

Group	Chlorophyll a	Chlorophyll b	Total carotenoid	β-carotene	Lycopene
Peel					
WT I	0.04 ± 0.004	0.07 ± 0.004	184.9 ± 5.66	1.10 ± 0.259	0.12 ± 0.044
Mut I	0.04 ± 0.001	0.07 ± 0.003	146.9 ± 3.11*	1.06 ± 0.239*	0.13 ± 0.051
WT II	0.04 ± 0.003	0.06 ± 0.002	193.0 ± 10.45	3.49 ± 0.203	nd†
Mut II	0.03 ± 0.001*	0.07 ± 0.005*	186.1 ± 8.35*	2.79 ± 0.414*	nd
WT III	0.04 ± 0.001	0.07 ± 0.003	231.4 ± 0.80	3.14 ± 0.304	nd
Mut III	0.04 ± 0.002	0.07 ± 0.005	187.1 ± 4.07*	1.47 ± 0.879*	0.35 ± 0.348
WT IV	0.04 ± 0.001	0.07 ± 0.003	241.7 ± 3.16	2.72 ± 0.455	nd
Mut IV	0.04 ± 0.002	0.07 ± 0.004	195.4 ± 1.90*	2.71 ± 0.482	nd
Pulp					
WT I	0.03 ± 0.001	0.07 ± 0.003	90.7 ± 0.99	0.53 ± 0.074	0.17 ± 0.014
Mut I	0.04 ± 0.002*	0.07 ± 0.006	70.6 ± 1.21*	0.93 ± 0.069*	0.22 ± 0.011*
WT II	0.03 ± 0.001	0.07 ± 0.004	91.4 ± 0.78	0.88 ± 0.304	0.28 ± 0.125
Mut II	0.03 ± 0.000	0.06 ± 0.001*	74.2 ± 0.48*	nd†	0.45 ± 0.444*
WT III	0.03 ± 0.002	0.06 ± 0.001	81.7 ± 1.57	nd	0.62 ± 0.350
Mut III	0.03 ± 0.001	0.07 ± 0.000*	55.9 ± 0.59*	0.52 ± 0.076	0.23 ± 0.011*
WT IV	0.03 ± 0.002	0.07 ± 0.002	101.8 ± 1.38	1.38 ± 0.201	0.13 ± 0.004
Mut IV	0.04 ± 0.003*	0.08 ± 0.006*	81.7 ± 0.76	nd	1.08 ± 0.309*

† Not detected; * Values are significantly different from corresponding WTs ($p < 0.05$).

from pale yellow to deep orange due to variations in type and quantity of different carotenoids (Scordino et al., 2011). Due to the presence of chlorophylls, immature fruits are capable of photosynthesis but cannot make significant contribution to own nutrition. There is a rapid synthesis of carotenoids in the chromoplast during ripening, which is accompanied by a simultaneous loss of chlorophylls (Scordino et al., 2011); in many citrus there is no further syn- thesis of colored carotenoids during ripening after all chlorophylls had disappeared (Scordino et al., 2011). The analyzed Mut peel and pulp samples contained in fact a higher amount of chlorophylls than those of WT (Table 2). Chlorophylls in citrus consist mainly of two pigments: chlorophyll a and chlorophyll b; chlorophyll c, d and chlorophyll e are not reported in citrus and are mainly present in algae and certain sea weed (Ladaniya, 2007; Scordino et al., 2011).

Further works are in progress in our laboratory to elucidate the identity of other bioactive compounds responsible for the potential health-promoting effects with citrus mutants obtained from radiation breeding programs.

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