Changes in the phenolic composition of citrus fruits and leaves prepared by gamma irradiation of budsticks

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Abstract: Citrus mutants were induced by the irradiation of citrus budsticks with 120 Gy of cobalt (^{60}CO) gamma irradiation. Three mutant plants demonstrating improved fruit quality were selected and compared with wild-type citrus plant for evaluation of the phenolic composition, such as total phenolics, total flavonoids, flavonoid distribution and D-limonene. The results show that irradiation induced changes in total phenolic and flavonoid contents of the fruit peel and pulp, and leaves of citrus mutants, as well as in D-limonene content. HPLC analysis demonstrated that hesperidine, narirutin and rutin were variably distributed in citrus mutants. The obtained results implicate that gamma irradiation may contribute to variations in phenolic composition.
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1. Introduction

Gamma irradiation has been used as an effective plant breeding method, which can greatly induce high mutation numbers and modify physiological characteristics to create new mutants with improved properties (Naito et al., 2005; Predieri, 2001; Sarto et al., 2006). Free radicals produced by gamma irradiation can cause changes in structure, function and metabolism of plant cells, which resultantly produce higher amounts of commercially useful metabolites and develop economically significant varieties (Kim et al., 2004; Wi et al., 2007). Many useful mutants were obtained by gamma irradiation at vegetative propagated plants (Ahloowalia and Maluszynski, 2001; Predieri, 2001).

Citrus fruits rank first in the world with respect to production among the fruits (Terol et al., 2010). The citrus crop is also a valuable contributor to the economy of Jeju island, Korea, with annual yields of more than 0.6 million tons. Citrus fruit production and market demand depends on several factors like life cycle of fruit-bearing trees, disease resistance, extended harvesting period, maximum yield of higher fruit qualities (e.g., size, color, texture and nutritional value of fruit) are important considerations. To meet the requirements of the fruit industry and the consumers, citrus mutation breeding approach via gamma ray irradiation to develop new citrus cultivars has steadily increased (Bermejo et al., 2011; Chaudhuri, 2002; Deng, 2005; Ling et al., 2008; Raza et al., 2003; Wu, 1986). However, limited information exists on the potential and extent of enhancing production of citrus secondary metabolites using gamma irradiation, even though many of the effects of gamma irradiation on seedlessness, pollen germination, fruit quality and growth of citrus have received considerable attention (Bermejo et al., 2011; Chaudhuri, 2002; Raza et al., 2003; Wu, 1986). In the present study, the levels of bioactive constituents were thus analyzed for non-irradiated and irradiation-induced citrus in order to compare their properties and changes.

2. Material and Methods

2.1. Gamma irradiation and plant maintenance

The citrus cultivar, *Citrus unshiu* Marc. cv. Miyagawa, which is most widely cultivated in Jeju island, Korea, was selected as the original plant materials for mutational breeding experiments. One year old shoots of citrus were exposed to gamma irradiation at the doses of 120 Gy from cobalt (^{60}CO) source at the Institute for Nuclear Science and Technology, Jeju National University and were then grafted onto one year old sour orange (*C. aurantium* L.) seedling. Afterwards, young irradiated tree were maintained for about 1 year at nursery and then selected mutant plants were moved to the main garden in Subtropical Horticulture Research Institute at Jeju National University where the rest of the study was carried out. Both citrus wild-type (WT) and mutant (Mut) plants were managed in same way with respect to fertilization, irrigation and disease control.

The growing conditions and degree of ripeness at harvest were controlled to eliminate the effects of environmental conditions on the different WT and Mut cultivars.

2.2. Sample preparation

Fully mature citrus fruits with leaves were harvested on ripening in November, 2010. Three different Mut cultivars were used in the present study. According to the morphological characteristics, including fruit shape, size and color, and contents of sugar and acid, which were determined in detail as described previously (Oh and Kim, 2011), test subjects were divided into four groups: WT, citrus derived from non-irradiated shoots; Mut I, citrus mutants with comparatively high sugar/acid ratio; Mut II, citrus mutants with red color; Mut III, citrus mutants with rough shape. The fruit peel and pulp, and leaves of WT and Mut citrus were separated, dissected, weighed, lyophilized and then ground into a fine powder using a blender. Portions (25 g) of the powered samples were successively extracted with 250 mL of methanol in the 25 ºC shaking incubator for 24 h and subsequently purified by using a 0.45 μm membrane filter (Waters, Milford, MA, US), which were stored at 4 ºC until further use.

2.3. Analysis of total phenolic and flavonoid contents

Contents of total phenol and flavonoid were determined by the modified method described previously (Senevirathne et al., 2010). For total phenol quantitation, 30 μL of citrus WT and Mut methanolic extracts were mixed thorough with 30 μL of 95% ethanol, 150 μL of distilled water, 15 μL of Folin-Ciocalteu reagent and 30 μL of saturated sodium carbonate solution (5%). After 60 min standing, the absorbance was read at 725 nm against a blank in a Spectra MR microplate reader (Dynex Technologies, Inc., Chantilly, VA, US). Content of phenols was calculated on the basis of the calibration curves of gallic acid, and was expressed as mg gallic acid per 100 g dry matter. To determine total flavonoids, each extract in methanol (15 μL) was mixed with 4.5 μL of 5% $NaNO_2$, 60 μL of distilled water and 4.5 μL of 10% $AlCl_3$. After incubation for 6 min, 60 μL of NaOH solution (4%) was added to the mixture and made up to a final volume of 150 μL with distilled water. The absorbance was measured 15 min later at 510 nm. Content of flavonoids was calculated on the basis of the calibration curves of rutin and was expressed as mg rutin per 100 g dry matter.

2.4. HPLC analysis of flavonoids

A 20 μL aliquot of citrus WT and Mut methanolic extracts was injected into a Shim-pack VP-ODS (C_{18}) column (4.6 m × 150 mm × 5 μm; Shimadzu, Tokyo, Japan) of the Waters high performance liquid chromatography (HPLC) system equipped with a 626 pump, a 486 UV-VIS detector and autosampler (Waters, Milford, MA) . The mobile phase for the HPLC system was (A) water/acetic acid (99.5/0.5, v/v): (B) acetonitrile/acetic acid (99.5/0.5, v/v) at a flow rate of 1 mL/min. The mobile phase program consisted of four periods: 0-10 min, 20% A; 10-16 min, 45% A; 16-20 min, 75% A; and 20-22 min, 20% A. The column was operated at 40 °C, and the eluent was monitored with a single-channel UV detector at a wavelength of 280 nm. The flavonoids were identified by comparing their retention times and UV spectra with those of authentic standards (ChromaDex, Irvine, CA, US). The content of each flavonoid was calculated from the integrated peak area if the sample and the corresponding standard.

2.5. GC-MS analysis of D-limonene

A Shimadzu QP2010 Ultra gas chromatographic mass spectrometric (GC-MS) system, autosampler and real time anlysis software were used for the analysis. A Restek Rtx-5MS capillary column (30 m × 0.25 mm i.d. × 0.25 μm) was used for separation. The injector temperature was 250˚C. The temperature program was started at 50 ˚C for 2 minute and heated at 10 ˚C/min to 80 ˚C, increased by 20 ˚C/min up to 140 ˚C and then by 40˚C /min up to 280 ˚C. Sample injection was done in split mode (split ratio 20:1). High purity helium was used as carrier gas at a flow rate of 1 mL/min. Mass spectrometry was run in full scan mode (m/z 45-500) with 200 ˚C MS source temperature and 2.5 minutes solvent cut time. D-limonene was indentified by comparing the retention times and mass spectra obtained with those of authentic standard (Sigma).

2.6. Statistical analysis

Results are presented as means ± standard deviation. Statistical comparisons were made by analysis of variance (ANOVA) procedure followed by a Duncan's multiple range tests (SPSS 12.0). $P < 0.05$ was considered significantly different. After multiple comparisons, the means in the following table and figures were followed with different small letter "a-d" based on their values and statistical differences.

3. Results and Discussion

Data in Figure 1 show that the Gamma irradiation significantly affected the total phenolic and flavonoid content of citrus when compared with

WT samples. The peel and pulp extracts of citrus Mut I and II had significantly higher total phenolic content than the extracts of citrus WT, whereas lower total phenolic content was noted for those of Mut III (Figure 1A). The citrus Mut I peel had the highest total phenolic content (23786 mg/100 g) which is 37% higher than that of WT peel (Figure 1A). Total flavonoid content in the peel (6766–7126 mg/100 g) and pulp (4874–5685 mg/100 g) of citrus WT, Mut I and Mut II were almost in the similar quantity (Figure 1B). However, the flavonoid contents of citrus Mut III peel and leaves were significantly higher than those of WT, while flavonoid content of Mut III pulp was significantly lower ($p < 0.05$) (Figure 1B). In this study, citrus peel and pulp extracts had relatively higher total phenolic and flavonoid contents as compared with the leaf extracts, except that of Mut III group (Figure 1).

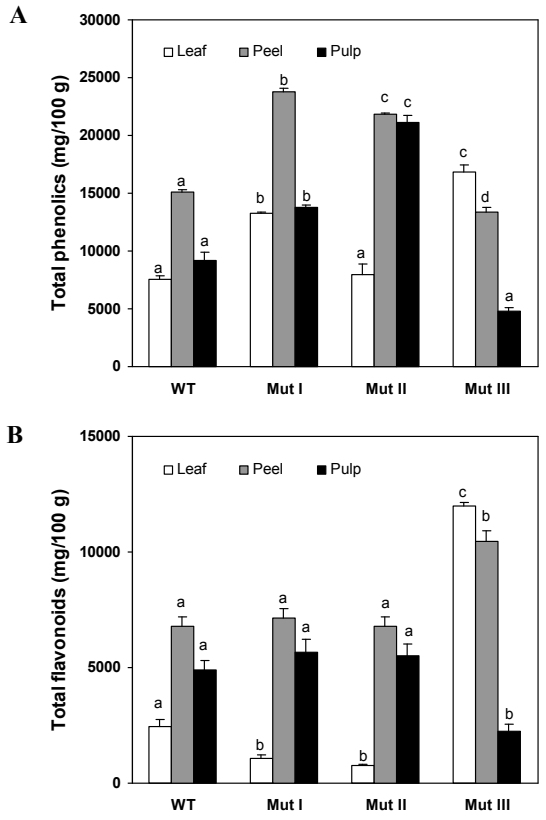

Fig. 1. Total phenolic (A) and flavonoid (B) contents in the leaves, peel and pulp extracts of citrus derived from non-irradiated and irradiated shoots. Data presented are in mean ± standard deviation ($n = 3$) which with different letters above the bars are significantly different at $p < 0.05$.

Flavonoids are a widely distributed group of phenolic compounds, which have a wide range of biological effects, such as inhibition of key enzymes in mitochondrial respiration, protection against coronary heart disease and anti-inflammatory, antitumor, and antimicrobial activities (Wang et al., 2008). Citrus fruit contains high levels of the flavanones, as well as flavonol, which are very rare in other plants (Gattuso et al., 2007). In the present study, four flavanones (naringin, neohesperidin, hesperidin and narirutin) and one flavonol (rutin) were identified in leaf extracts, while hesperidin, narirutin and rutin were the main flavonoids detected in citrus peel and pulp extracts (Table 1). The amounts of hesperidin in peel (340.3-573.7 mg/100 g), pulp (105.4-276.3 mg/100 g) and leaves (248.3-829.5 mg/100 g) of citrus in this work were the highest compared to amounts of other flavonoids. The amounts of naringin, hesperidin and rutin were significantly lower but narirutin was significantly higher in leaf extracts of all Mut groups than WT ($p < 0.05$). Neohesperidin occurred in the leaf extracts of all experimental groups with similar distribution patterns (42.0-43.3 mg/100 g) (Table 1). Here, obtained results of peel and pulp showed that these two parts had very similar flavonoids tendencies without remarkable differences although peel contained higher level of investigated flavanones than pulp. The peel and pulp of all Mut groups (78.5-93.7 and 93.4-132.2 mg/100 g, respectively) contained significantly higher concentration of narirutin than those of WT (60.8 and 92.4 mg/100 g, respectively); In the peel and pulp of Mut III, the flavanone glycoside pattern consisted predominantly of hesperidin and narirutin, and their contents were significantly higher than WT ($p < 0.05$). Hesperidin has been reported to reduce plasma/hepatic cholesterol (Montforte et al, 1995; Manach et al, 2003) and to suppress the oxidative stress *in vitro and vivo* (Miyake et al, 1998). Furthermore, it has been shown to protect animals against chemically induced several cancers (Tanaka et al, 1997; Berkarda et al, 1998). Narirutin has been demonstrated to have an anti-allergic properties (Kubo et al, 2004) and therapeutic effect on bronchial asthma (Funaguchi et al, 2007).

Citrus oils are mixtures of volatile components of terpenic hydrocarbon and oxygenated compounds (Cano and Bermejo, 2011). D-limonene is the principle component of citrus oil, ranging from 88% to 95%, which is often used as an additive in food products and fragrances, natural pesticide and insect repellent (Cano and Bermejo, 2011). It has also been studied for its anti-carcinogenic properties (Crowell and Gould, 1994).

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

Group	Flavanone						Flavonol glyside
	Naringin	Neohesperidin	Hesperidin	Narirutin	Naringenin	Hesperetin	Rutin
Leaves							
WT	30.9±3.04[a]	43.3±1.43	829.5±1.86[a]	18.8 ± 0.65[a]	nd[*]	nd	254.1±2.07[a]
Mut I	20.6±1.43[b]	42.0±1.66	292.9±2.02[b]	22.0 ± 1.16[b]	nd	nd	186.6±1.76[b]
Mut II	21.7±1.13[b]	42.0±2.50	293.9±1.47[b]	26.8 ± 0.91[c]	nd	nd	134.1±2.72[c]
Mut III	17.4±0.48[c]	43.3±0.79	248.3±1.29[d]	23.1 ± 0.30[b]	nd	nd	186.4±0.99[b]
Peel							
WT	nd	nd	463.1±1.11[a]	60.8±1.51[a]	nd	nd	65.7±0.47[a]
Mut I	nd	nd	573.7±3.71[b]	93.7±0.61[b]	nd	nd	72.1±0.93[b]
Mut II	nd	nd	340.3±5.94[c]	111.3±1.23[c]	nd	nd	64.0±0.87[c]
Mut III	nd	nd	546.6±3.38[d]	78.5±0.61[d]	nd	nd	66.1±0.63[a]
Pulp							
WT	nd	nd	178.8±0.43[a]	92.4 ± 0.59[a]	nd	nd	65.7 ± 0.47[a]
Mut I	nd	nd	145.1±1.35[b]	93.4 ± 0.16[a]	nd	nd	72.1 ± 0.93[b]
Mut II	nd	nd	105.4±0.67[c]	131.9 ± 0.60[b]	nd	nd	64.0 ± 0.87[c]
Mut III	nd	nd	276.3±2.07[d]	132.2 ± 1.53[b]	nd	nd	66.1 ± 0.63[a]

[*] Not detected; [a-d] Values with different superscripts in a column are significantly different ($p < 0.05$)

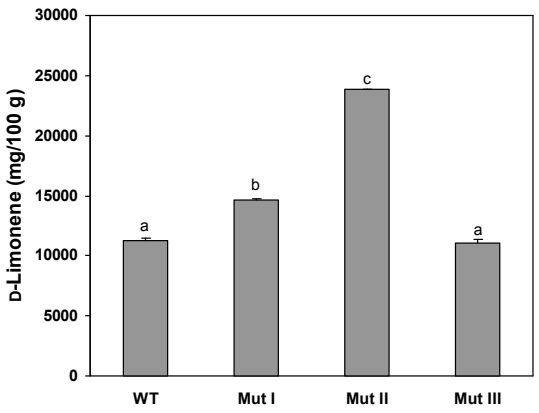

Fig. 2. D-limonene content in the leaves (A) and peel (B) extracts of citrus derived from non-irradiated and irradiated shoots. Data presented are in mean ± standard deviation ($n = 3$) which with different letters above the bars are significantly different at $p < 0.05$.

The gas chromatographic profiles and D-limonene content are presented in Figure 2 in values of milligrams per 100 gram of peel (dried weight). Mut I (14679 mg/100 g) and II (23844 mg/100 g) displayed significantly higher D-limonene values than WT (11222 mg/100 g) ($p < 0.05$). However, Mut III (11108 mg/100 g) showed similar content with WT with no significant differences (Figure 2).

In this current study, we examined the influence of gamma irradiation on the phenolic constituents of citrus. Overall, gamma irradiation causes biochemical changes in the fruits and leaves, and seem to be able to influence the synthesis of phenolic compounds, which have been recognized as important due to their bioactive role and heath benefits although it would be necessary to carry out more analyses, and with citrus mutants obtained from radiation breeding programs, to elucidate conclusive values.

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