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The improvement of ginsenoside accumulation in *Panax ginseng* as a result of γ-irradiation

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In this study, gamma rays were used to irradiate embryogenic calli induced from cotyledon explants of *Panax ginseng* Meyer. After the embryogenic calli were irradiated, they were transferred to adventitious roots using an induction medium; next, mutated adventitious root (MAR) lines with a high frequency of adventitious root formations were selected. Two MAR lines (MAR 5-2 and MAR 5-9) from the calli treated with 50 Gy of gamma rays were cultured on an NH_4NO_3-free Murashige and Skoog medium with indole-3-butyric acid 3 mg/L. The expression of genes related to ginsenoside biosynthesis was analyzed using reverse transcription polymerase chain reaction with RNA prepared from native ginseng (NG), non-irradiated adventitious root (NAR) and 2 MAR lines. The expression of the squalene epoxidase and dammarenediol synthase genes was increased in the MAR 5-2 line, whereas the phytosterol synthase was increased in the MAR 5-9 line. The content and pattern of major ginsenosides (Rb1, Rb2, Rc, Rd, Re, Rf, and Rg1) were analyzed in the NG, NAR, and 2 MAR lines (MAR 5-2 and MAR 5-9) using TLC and HPLC. In the TLC analysis, the ginsenoside patterns in the NG, NAR, and 2 MAR lines were similar; in contrast, the MAR 5-9 line showed strong bands of primary ginsenosides. In the HPLC analysis, compared with the NG, one new type of ginsenoside was observed in the NAR and 2 MAR lines, and another new type of ginsenoside was observed in the 2 MAR lines irradiated with gamma rays. The ginsenoside content of the MAR 5-9 line was significantly greater in comparison to the NG.

Keywords: *Panax ginseng*, Gamma irradiation, Ginsenoside, HPLC, TLC

INTRODUCTION

Panax ginseng Meyer is a perennial herb that belongs to the family Araliceae. Ginseng root is a well-known oriental herb that has been widely used for health food and traditional medicine since ancient times. Various pharmacological effects of ginseng have been reported, such as improved immunity, stamina, health and enhanced resistance to stress [1-4]. Recent research has confirmed the medicinal properties and pharmacological potential of the ginseng root [5-8], thereby increasing global interest in its use. The primary pharmacologically active ingredients in ginseng are ginsenosides, which are

triterpene saponins [9]. Ginsenosides are a secondary metabolite of ginseng and are classified into two groups based on their aglycone structure, namely, the dammarane and oleanane types. The most common ginsenoside is the dammarane type, which can be divided into Rb (Rb1, Rb2, Rc, and Rd) and Rg (Re, Rf, and Rg1) groups by the difference in the 20(S)-protopanaxadiol and 20(S)-protopanaxatriol structure, respectively [10-12]. The oleanane-type ginsenosides are the only R0 in which the fundamental skeletons are pentacyclic. More than 30 kinds of ginsenosides, such as Rg2, Rf1 and Rh2, have

been isolated and studied [6,7].

Although ginseng has many beneficial pharmacological properties, native ginseng takes a long time to reach maturity (4 to 6 years), cultivation is risky, and ginsenoside production is minimal. Therefore, mass production of ginseng is needed in order to meet demand. As a result of these challenges, many reports have been published concerning ginsenoside production using transformed roots, adding biotic or abiotic elicitors in cell cultures, and producing large-scale cultures with bioreactors [13-16]. Tissue culture is particularly important for the mass production of ginseng, and the quality of culture is highly dependent on the culture media and hormone composition. In numerous other studies, ginseng culture techniques, such as callus induction, cell suspension, adventitious and hairy root induction, and optical culture conditions, such as the addition of elicitors and hormones for mass production, have been evaluated [17-20].

The term 'somaclonal variation' characterizes the process of induced genetic variation by *in vitro* culture. Somaclonal variations are attributable to point mutation, chromosomal rearrangements, and DNA methylation [21,22]. Somaclonal mutants have provided valuable genetic resources for plant breeding. However, although it is possible to select mutants by somaclonal variation, mutant frequencies are often low compared with artificial mutagenesis employing such methods as ionizing radiation and chemical treatments [23].

Ionizing radiation can affect plants in several ways, such as by damaging DNA, altering bases and sugars, forming DNA-DNA and DNA-protein crosslinks, and by causing single strand breaks and double-strand breaks [24,25]. Numerous researchers have recently performed mutation breeding by ionizing radiation. Kim et al. [26] reported that high levels of amino acids were obtained from mutant rice lines. The transcript levels of the putative *OASA2* mutant gene in these mutant lines were higher than in the control. Also, according to Lee et al. [27], the radiation-induced gene mutations within the anthocyanin pathway are associated with variations in the color of chrysanthemum flowers. In addition, new and improved varieties of many crops, such as cocoa, potato, banana, and sugarcane, have been developed [28-31].

In this study, we induced adventitious roots from the cotyledon of ginseng that had been mutagenized with gamma rays. Next, we selected mutant lines showing high-growth performance. We analyzed the expression patterns of genes related to triterpene biosynthesis in the mutated adventitious root (MAR) lines compared with the native ginseng (NG) and non-irradiated adventitious root (NAR) lines. In addition, we profiled and qualified ginsenosides using TLC and HPLC systems.

MATERIALS AND METHODS

Plant materials

Callus cell cultures were induced on a Murashige and Skoog (MS) medium (pH 5.8); the cultures were subsequently supplemented with 1 mg/L of 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.1 mg/L of kinetin at 25°C in a dark environment. The calli of *P. ginseng* were irradiated with 50 Gy of a cobalt-60 gamma-irradiator (150 TBq of capacity; ACEL, Ottawa, ON, Canada) at the Korea Atomic Energy Research Institute.

Adventitious roots were formed on a solid NH_4NO_3-free MS medium and supplemented with 3 mg/L of inodole-3-butyric acid (IBA) at 23°C in a dark environment [32]. The fresh mass of adventitious roots was measured after 4 wk. The liquid culture was maintained in an NH_4NO_3-free MS medium supplemented with 3 mg/L of IBA on a gyratory shaker (100 rpm) under the same conditions.

Reverse transcription polymerase chain reaction analysis

After 4 wk of culture, the total RNA from 0.1 g of *P. ginseng* hairy roots was isolated using an easy-spin Plant RNA Extraction Kit (Intron Biotechnology, Seoul, Korea). For reverse transcription, 1 μg of RNA was subjected to cDNA synthesis in 10 μL of reaction mixture. Reverse transcription (RT) reactions were performed using 0.25 U of avian myeloblastosis virus (AMV) reverse transcriptase in 1 U of RNase inhibitor, 1 mM dNTP, 5 mM $MgCl_2$, 1×RT buffer, and oligo (dT) 15 primers at 42°C for 60 min. The reaction was terminated by heating the mixture at 70°C for 10 min followed by cooling at 4°C. The resulting cDNA served as a template for subsequent polymerase chain reaction (PCR) amplification using primers for the ginsenoside synthesis genes (Table 1). The PCR amplification conditions involved 3 mM at 94°C for the initial denaturation followed by 25 cycles of the following: 30 s at 94°C, 30 s each at 58°C, and 30 s at 72°C followed by 5 min at 72°C for the final extension. The sizes of the PCR products were determined using electrophoresis in an agarose gel (1.2%).

Extraction of ginsenosides

The extraction of ginsenosides was performed using the methods described in Woo et al. [15]. Milled powder (0.5 g) from freeze-dried adventitious roots was soaked

Table 1. The primers of ginsenoside synthetic genes for the reverse transcription polymerase chain reaction analysis

Oligo name	Sequence	Size (bp)
PgSE (1)	F: ATGGGAAGTTTGGGGGCAATTCT	1,252
	R: GTTCTCACTGTTTGTTCAGTAGTAGGTT	
PgSE (2)	F: AGCAGCAGTTGACAAAGG	506
	R: GCCACATTCGTTTTGGTGAAGG	
PNX (3)	F: TCATCAGATGGCTCATGGTACG	361
	R: TCTCCTCCTGTGGGAAATCACC	
PNY (4)	F: TATCCTGGACACCGAAAGAAGG	445
	R: CTCCACTTATTTCCTGTTGGGG	
PNY2 (5)	F: TGGATTTTCTAATAAAATCGCAACGCAG	425
	R: TTTCATTTGAGTATTGGCAGGCCG	
PgDDS (6)	F: ATGTGGAAGCTGAAGGTTGCTCAAGGA	2,310
	R: TTAAATTTTGAGCTGCTGGTGCTTAGGC	

PgSE, squalene epoxidase; F, forward; R, reverse; *PNX*, cycloartenol synthase; *PNY*, oxidosqualene cyclase; *PNY2*, oxidosqualene cyclase 2; *PgDDS*, dammarenediol synthase.

in 80% MeOH at 40°C. After the liquid evaporated, the residue was dissolved in H_2O and washed with $(C_2H_5)_2O$. Then, the residue was extracted with H_2O-saturated *n*-butanol and washed twice with H_2O. The *n*-butanol layer was subsequently evaporated to produce the ginsenoside fraction. Next, each sample was dissolved in MeOH (HPLC-grade) and filtered once through a 0.2-μm nylon filter.

TLC identification of individual ginsenosides

The ginsenoside fraction was analyzed using a TLC system. The TLC analysis was conducted using automated multiple development (AMD; Camag, Muttenz, Switzerland). The ginseng extracts (25 mg) in methanol were sprayed onto a TLC plate (silica gel, 10 cm×20 cm). The mobile phase was conducted using a solvent system (chloroform:methanol:water=65:35:10) and a migration distance of 80 mm. The ginsenosides were subsequently imaged with brown bands by applying 10% H_2SO_4 and heating at 120°C.

HPLC analysis

The HPLC conditions were based on those described in Court et al. [33], which provided satisfactory resolution of the seven major ginsenosides, Rb1, Rb2, Rc, Rd, Re, Rf, and Rg1. The ginsenoside separation was conducted on a Zorbax 300SB-C18 analytical fractions column (particle size 5 μm, 4.6 mm×150 mm; Agilent, Santa Clara, CA, USA) using the following gradient system: 0 to 10 min, 10% acetonitrile and 90% distilled water (DW); 10 to 45 min, 50% acetonitrile and 50% DW; 45 to 55 min, and 90% acetonitrile and 10% DW.

The flow rate was 1 mL/min, and the ginsenosides were monitored at 203 nm. The sample injection quantity was 15 μL, and the temperature of the column was sustained at 30°C. The ginsenoside peaks were monitored, with the peak areas corresponding to samples matching authentic ginsenoside standards (Rb1, Rb2, Rc, Rd, Re, Rf, and Rg1) purchased from ChromaDex (Santa Anna, CA, USA). The data were compared with an external standard calibration curve.

RESULTS AND DISCUSSION

Selection of mutated adventitious root lines

To obtain the MAR lines, the ginseng seed was germinated on a MS media supplemented with 1 mg/L 2,4-D and 0.1 mg/L Kinetin. An amorphous embryogenic callus was induced from ginseng cotyledons and was subcultured on the same medium (Fig. 1A, B). The embryogenic callus was irradiated with 50 Gy of gamma rays (from a ^{60}Co source). After irradiation, the embryogenic callus was transferred to an adventitious root induction solid from an NH_4NO_3-free medium supplemented with IBA 3 mg/L; next, mutated adventitious root lines showing high growth performance were selected (Fig. 1C, D).

In our previous report, the highest frequency of adventurous root formation was observed on the NH_4NO_3-free MS medium with IBA 3 mg/L when compared with the medium from hormones 2,4-D and naphthalene acetic acid [34,35]. The fresh mass was confirmed to be greater from a 50 Gy treatment compared with the fresh mass resulting from the non-irradiated callus [32]. We selected 2 MAR lines (MAR 5-2 and MAR 5-9) from the calli
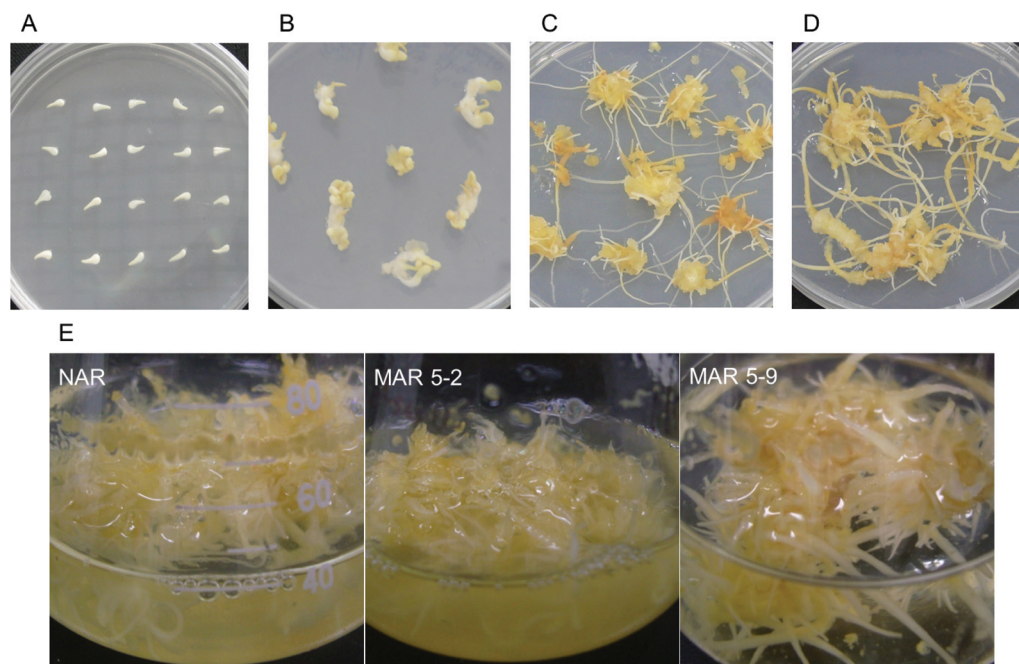
Fig. 1. Adventitious root production from gamma irradiated mutant lines: (A) ginseng seed was germinated by *in vitro* culture, (B) amorphous embryogenic calli were induced from ginseng cotyledons, (C) embryogenic callus formation on the callus induction medium, (D) selected high frequency of adventitious root formation, (E) proliferation of the adventitious root in the liquid adventitious root induction medium. NAR, non-irradiated adventitious root; MAR, mutated adventitious root (5-line).
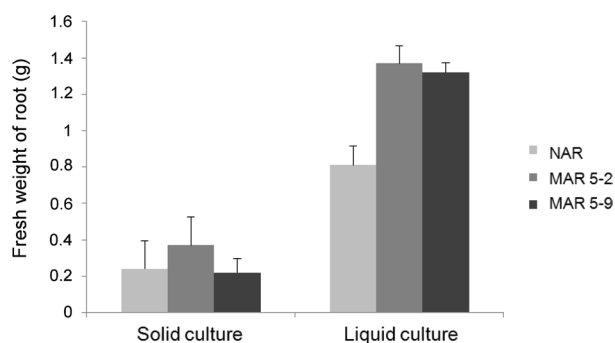

Fig. 2. The fresh mass of adventitious roots of the non-irradiated adventitious root (NAR) and 2 mutated adventitious root (MAR) lines after 4 wk.

treated with 50 Gy of gamma rays. These lines were propagated on the NH_4NO_3-free MS medium with IBA 3 mg/L (Fig. 1E). The fresh mass of the 2 MAR lines (MAR 5-2 and MAR 5-9) was compared with the NAR line after the liquid and solid cultures were incubated for 4 wk. The fresh weight of the MAR 5-9 line was similar to that of the NAR line, whereas the MAR 5-2 line was greater than the NAR line in the solid culture. In contrast, the growth of the 2 MAR lines was much greater than that of the NAR line in the liquid culture (Fig. 2).

Tissue culture techniques influence the growth of plant mass production. Also, there can be various outcomes from mutation breeding by irradiation. Each spectrum of γ-irradiation has a different effect on plant growth [29,32].

Expression of ginsenoside biosynthesis

Ginsenosides, which are glycosylated triterpenes and triterpenes, are biosynthesized by the mevalonate pathway in the cytosol (Fig. 3). The expression of genes related to ginsenoside biosynthesis (Table 1) was examined by RT-PCR analysis with RNA prepared from a 6-year-old native ginseng plant (naturally grown ginseng, NG) and from an NAR and 2 MAR lines (Figs. 3 and 4). The transcription levels of *PgSE* and *PgSE* genes were increased in the NAR and 2 MAR lines compared with the NG line. The *PgSE* gene was highly expressive in the MAR 5-9 line, whereas the *PgSE* gene was highly expressive in the MAR 5-2 line. The *PgSE* and *PgSE* genes catalyzed the first step at creating ginsenoside biosynthesis pathways that regulate the production of phytosterols and triterpenoids in ginseng [36,37]. Three types of ginsenosides were synthesized from oxidosqualene, by cylization of oxidosqualene cyclases (OSC) genes: dammarenediol synthase (DDS), phytosterol synthase (*PNX*), and oleanane-type synthase (PNY1, PNY2). The expression of OSC genes, except for PNY1, was increased,
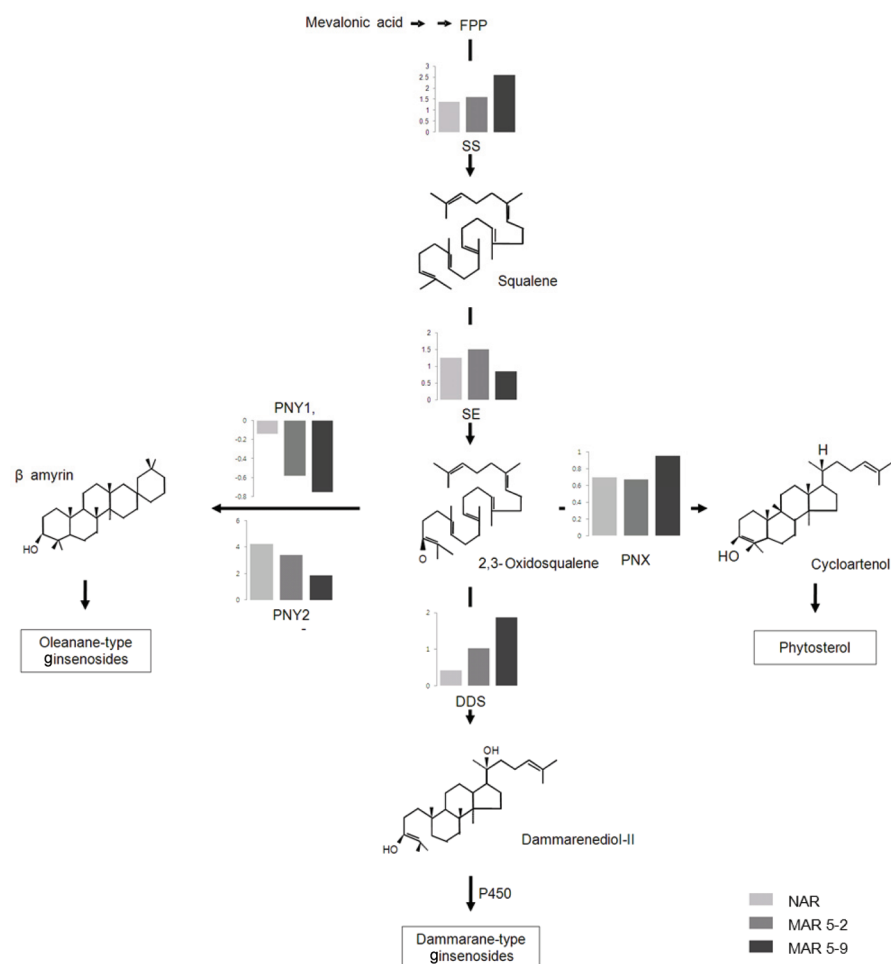
Fig. 3. The biosynthetic pathway of ginsenoside in *Panax ginseng*. FPP, farnesyl diphosphate; SS, squalene synthase; SE, squalene epoxidase; *PNX*, cycloartenol synthase; PNY1, oxidosqualene cyclase 1; PNY2, oxidosqualene cyclase 2; *DDS*, dammarenediol synthase; NAR, non-irradiated adventitious root; MAR, mutated adventitious root.
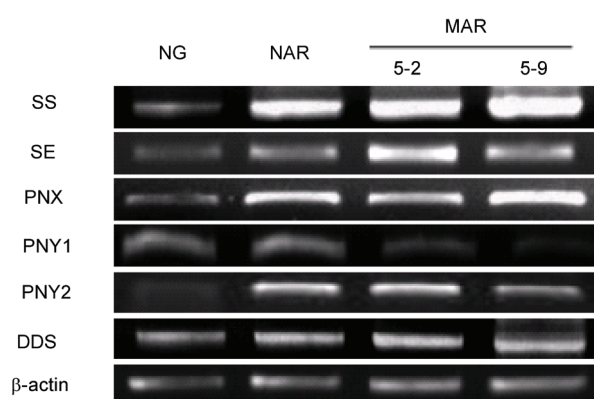

Fig. 4. The expression of genes related to ginsenoside biosynthesis in the mutated adventitious root (MAR). NG, native ginseng; NAR, non-irradiated adventitious root; SS, squalene synthase; SE, squalene epoxidase; PNX, cycloartenol synthase; PNY1, oxidosqualene cyclase 1; PNY2, oxidosqualene cyclase 2; DDS, dammarenediol synthase.

whereas the expression of PNY1 was decreased slightly compared with the NG. The *PNX* and *DDS* genes were the most highly expressive in the MAR 5-9 line. The OSC genes are involved in the biosynthesis of phytosterol synthase, oleanane-type synthase, and dammarenediol synthase [38,39]. The *DDS* gene was involved in the synthesis of ginsenoside, indicating that the *DDS* gene was highly expressive in the MAR 5-9 line [37]. These results indicate that the ginsenosides are more expressive in the MAR 5-9 line than the other samples.

Ginsenoside analysis by TLC and HPLC

Quantitative and qualitative variation of the seven major ginsenosides of the Rb1, Rb2, Rc, Rd, Re, Rf, and Rg types have been compared with the NG, NAR, and 2 MAR lines. To analyze the ginsenoside pattern, the extract of the NG, NAR, and 2 MAR lines were loaded on preparative silica gel TLC plates (Fig. 5A). The TLC
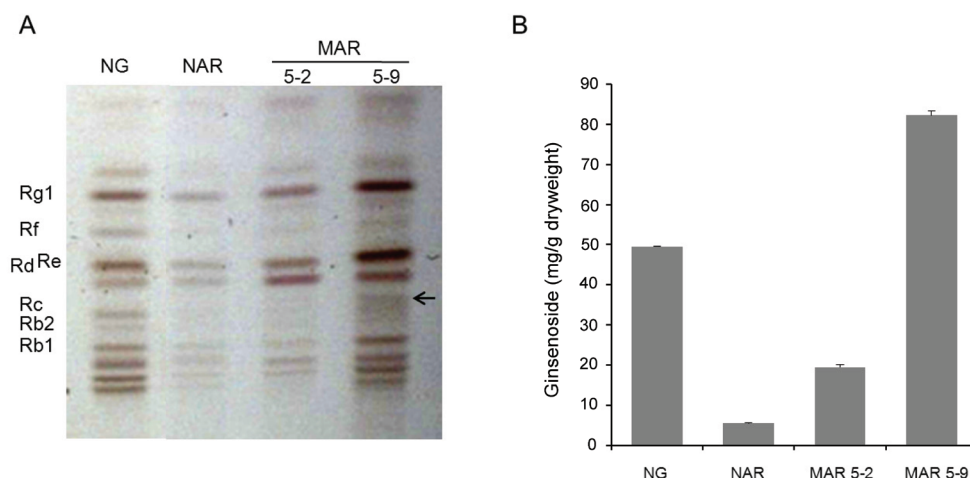
Fig. 5. A ginsenoside analysis using TLC and HPLC. (A) TLC analysis of ginsenosides. The star indicates a new type of ginsenoside. (B) Ginsenoside contents in the native ginseng (NG), non-irradiated adventitious root (NAR) and 2 mutated adventitious root (MAR) lines. Black arrow was a new band.

Table 2. Contents of ginsenosides in the NG, NAR, and 2 MAR lines

Line	Protopanaxadiol				Protopanaxatriol			Sum
	Rb1	Rb2	Rc	Rd	Re	Rf	Rg1	
NG	8.81±0.04	14.01±0.06	4.15±0.01	-	9.11±0.05	4.14±0.01	9.29±0.01	49.50±0.02
NAR	0.77±0.02	-	-	-	1.71±0.00	0.80±0.03	2.20±0.01	5.48±0.06
MAR 5-2	0.92±0.03	4.74±0.01	1.80±0.62	0.59±0.44	5.49±0.03	3.55±0.01	2.37±0.01	19.46±0.58
MAR 5-9	4.05±0.02	15.64±0.05	2.79±0.03	1.74±1.31	26.08±0.13	16.48±0.05	15.43±0.05	82.22±1.12

NG, native ginseng; NAR, non-irradiated adventitious root; MAR, mutated adventitious root.

revealed that the ginsenoside pattern was similar in the NG, NAR, and 2 MAR lines, although the bands of the NAR line and the MAR 5-2 line were smears. In contrast, the MAR 5-9 line was strongly represented in most of the bands compared with the NG. Also, the MAR 5-9 showed a new band under the Rd (Fig. 5A).

The ginsenoside content was determined from the roots of the NG, NAR, and 2 MAR lines. Compared with the NG, the amount of 7 ginsenosides of the MAR 5-9 line was 1.7-fold greater (Fig. 5B). The peaks were determined based on the retention times of the primary ginsenoside standard (Fig. 6). Among the 2 MAR lines, the MAR 5-9 line showed 4 times the ginsenoside content. The HPLC chromatogram revealed that the pattern of HPLC peaks was similar in the NG, NAR, and 2 MAR lines, although there were remarkable quantitative differences in each sample. The MAR 5-9 line showed greater quantities overall compared with the other samples. More ginsenosides were detected form the NG than form the NAR and MAR -5-2 lines.; however, the Rd ginsenoside from the NG line and the Rb2, Rc and Rd ginsenosides from the NAR line were undetectable in the HPLC. The

MAR 5-9 line and the NG line were compared: relative to the NG, the Rb1 and Rc ginsenosides of the protopanxadiol group decreased in 2-fold, whereas Re, Rf, and Rg1 ginsenosides of the protopanaxatirol group increased in 2.5-fold, respectively (Table 2). Furthermore, all of the ginsenoside content detected in the NAR and MAR 5-2 lines was low. In the previous report, the amount of 7 ginsenosides was higher in the NG callus culture [40,41]. In this paper, the ginsenoside content in the NG appeared to be greater than that of all of the samples, except for the MAR 5-2 lines. The enhanced accumulation of the ginsenoside content in the MAR 5-9 line suggests a significant effect of γ-irradiation on ginsenoside biosynthesis. The ginsenoside analysis of the NAR and 2 MAR lines showed different ginsenoside profiles compared to the NG. Also, the 2 MAR lines showed different ginsenoside patterns with those of the NAR (Fig. 6). We suggested that the differentiation of ginsenoside content among NAR and 2 MAR lines was caused by differential expression of genes related to the ginsenoside biosynthesis. The synthetic genes of dammarane-type ginsenosides in 2 MAR lines showed higher expression level than
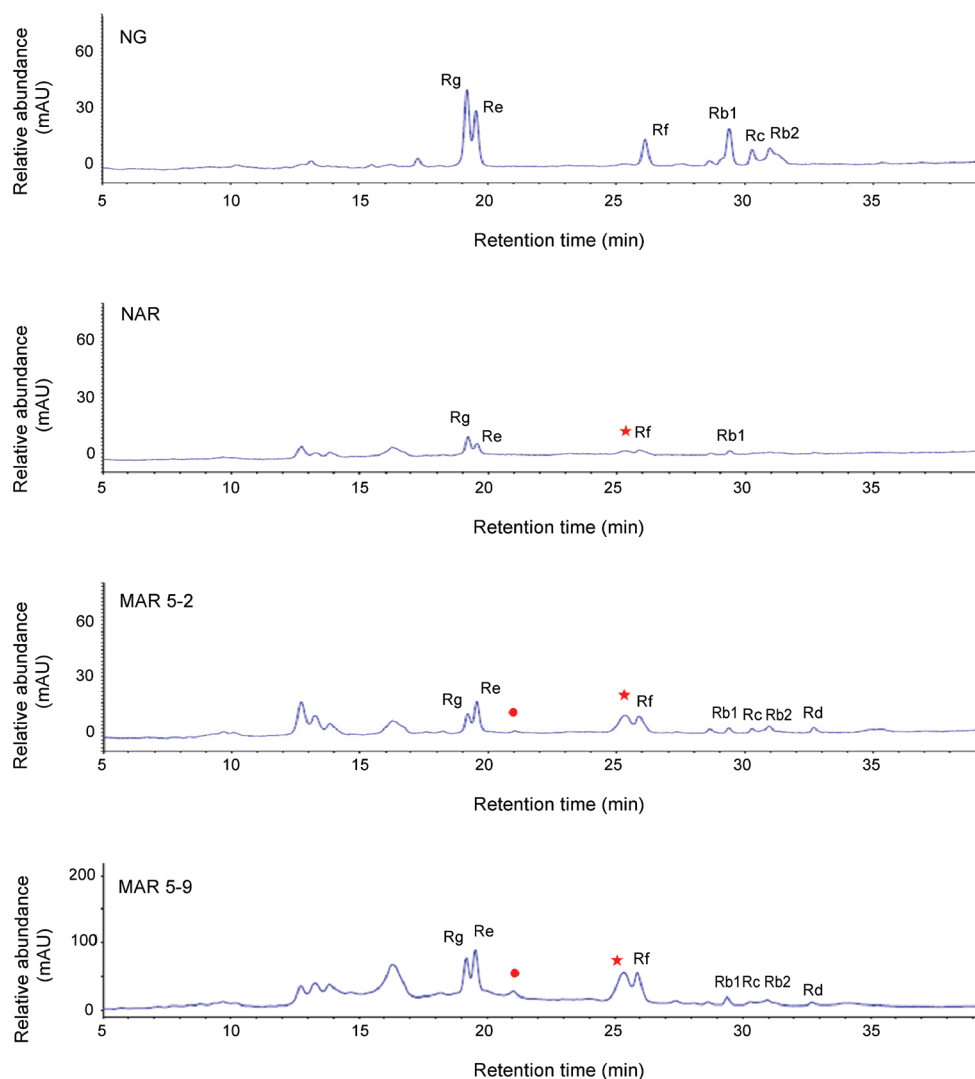
Fig. 6. The HPLC chromatograms of ginseng extracts in the non-irradiated adventitious root (NAR) and the 2 mutated adventitious root (MAR) lines. Seven ginsenosides including the Rb1, Rb2, Rc, and Rd of the Rb groups and the Re, Rf and Rg1 of the Rg group, were marked at each peak area. The star indicates a new type ginsenoside. NG, native ginseng.

NAR line, expect *PgSE* in MAR 5-9 (Fig. 3). Kim et al. [42] reported that the change of tryptophan synthesis key enzyme in mutant rice caused the change of tryptophan content. We seemed that the ginsenoside content in MAR 5-9 line was the highest than MAR 5-2 and NAR because of the highest level of *PgDDS* (Table 2 and Fig. 3).

The pattern and content of ginsenosides appeared differently in each γ-irradiated sample, suggesting that there was different enzyme activity involved in the ginsenoside pathways and culture conditions of each sample [10,34].

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