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Improvement of ginsenoside production by *Panax ginseng* adventitious roots induced by γ-irradiation

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Abstract

In order to evaluate effects of γ-rays on adventitious root formation and ginsenoside production, embryogenic calli induced from cotyledon explants of *Panax ginseng* C.A. Meyer were treated with γ-rays of 0, 10, 30, 50, 70, and 100 Gy. The highest frequency of adventitious root formation of 75 % occurred at γ-irradiation of 30 Gy, which is considered adequate dosage for selecting mutant cell lines. Five mutated adventitious roots (MAR)3-lines out of the propagation of 142 adventitious root lines treated with 30 Gy were selected based a 100-fold increase in proliferation rate compared to control adventitious roots (CAR) and content of the seven major ginsenosides (Rb_1, Rb_2, Rc, Rd, Re, Rf, and Rg_1) was determined. In the CAR and four of the MAR3-lines (except for MAR3-109), the Rb/Rg ratio was greater than 1.0, thereby indicating altered ginsenoside composition in these root lines. The HPLC analysis of the MAR3-13 and MAR3-26 lines confirmed different ginsenoside profiles, including the three unidentified ginsenoside candidates, Gm_1, Gm_2, and Gm_3. The ginsenosides of the MAR3-13 and MAR3-26 lines showed high hydroxyl and superoxide radical scavenging activities.

Additional key words: HPLC, mutation, radical scavenging activities.

Introduction

Panax ginseng C.A. Meyer is a traditional medicinal herb. The major pharmacologically active components are the ginsenosides, which are dammarane type triterpene glycosides containing a tetracyclic aglycone (Shibata *et al*. 1965). Over 32 types of ginsenosides have been identified among the species of genus *Panax* (Shibata 2001). Most ginsenosides exhibiting four forms of aglycone moiety: protopanaxadiol, protopanaxatriol, ocotillol and olenolic acid types (Fuzzati *et al*. 1999). The two major groups of ginsenoside are Rb and Rg. The major active ginsenosides of the Rb group have been identified as Rb_1, Rb_2, Rc, and Rd, while the Rg group contains Re, Rf, and Rg_1 (Tanaka and Kasai 1983, Smolenskaya *et al*. 2007).

In order to achieve a more rapid and increased production of the ginsenosides, the use of bioreactors for *in vitro* cultivation of ginseng cell and root cultures has been considered as alternative to field-cultivated native ginseng (Choi *et al*. 2003, Kim *et al*. 2004, Langhansová *et al*. 2005). Despite the promise of the bioreactors for improved production, root biomass yield have been found to be low presumably because of low growth rate in culture.

The increased production of ginsenosides, through an integration of a T-DNA fragment into the plant genome (Yoshikawa and Furuya 1987, Washida *et al*. 1998, Mallol *et al*. 2001, Palazón *et al*. 2003) or the addition of biotic or abiotic elicitors in cell cultures (Yu *et al*. 2002, Kim *et al*. 2004, Ali *et al*. 2006), has been extensively studied.

In vitro cultures revealed a rich array of culture-induced genetic variants, termed somaclonal variations. Many unstable or nontransmissible variants may have their origin in epigenetic rather than genetic changes

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Abbreviations: 2,4-D - 2,4 dichlorophenoxyacetic acid; CAR - control adventitious root; CL - chemiluminescence; IBA - indole-3-butyric acid; MAR - mutated adventitious root; MS - Murashige and Skoog; NAA - naphthaleneacetic acid.
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(Phillips *et al*. 1994, Chowdari *et al*. 1998, Yang *et al*. 1999). Somaclonal variations may include chromosomal rearrangements, single-gene mutants (mostly recessive), gene modifications and deletions, and DNA methylation (Larkin and Scowcroft 1981). Although it is possible to select desirable mutants, the mutation frequencies are often low. The major use of mutagenic treatment in *in vitro* cultures is to add a single allele at a time to an existing genotype and the multiplication of the desired genotype in a minimal space and time.

Although successful selection of mutants after *in vitro* application of ionizing radiation and *in vitro* mutagenesis efficiency has been reported in many major crops (Muthusamy *et al*. 2007), there are no reports on inducing mutations embryogenic callus of ginseng. In this paper, we have described the effects of hormone on the induction of embryogenic callus and adventitious roots and the selection of mutated adventitious root lines with high biomass productivity and ginsenoside content.

Materials and methods

Embryogenic callus induction and γ-irradiation: To induce embryogenic callus, *Panax ginseng* C.A. Meyer mature seed embryos were placed onto a solid callus induction Murashige and Skoog (MS) medium (pH 5.8) supplemented with 1 mg dm^{-3} 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.1 mg dm^{-3} kinetin. All cultures were incubated at 25 °C in the dark. The induced calli were exposed to different doses of γ-radiation (10, 30, 50, 70, and 100 Gy) emitted from a ^{60}Co source at a dose rate of 4.17 Gy h^{-1}. The γ-irradiation was conducted in the Korea Atomic Energy Research Institute. Effects of an irradiation on adventitious root formation was evaluated by measuring the frequency of formation and the fresh mass of roots after 40 d in the adventitious root induction medium.

Adventitious root formation and suspension culture: To examine the effects of indole-3-butyric acid (IBA), naphthaleneacetic acid (NAA), and 2,4-D on adventitious root formation, proliferating calli were placed on a solid NH_4NO_3-free MS medium supplemented with different concentrations of auxins (0, 0.1, 0.3, 1, 3, and 5 mg dm^{-3}). Five Petri dishes with 20 calli (5 - 10 mg fresh mass) were cultured per treatment and the experiment repeated three times. The adventitious root formation was examined after 4 weeks of culture. The adventitious roots were then transferred to a liquid NH_4NO_3-free MS medium supplemented with 3 mg dm^{-3} IBA on a gyratory shaker (100 rpm) at 23 ± 2 °C in the dark. The fresh mass was measured after 4 and 8 weeks of culture.

Extraction and analysis of ginsenoside by HPLC: The extraction of ginsenosides was performed according to Woo *et al*. (2004) with modifications. Milled powder (100 mg) from freeze-dried adventitious roots was soaked in 80 % MeOH for 180 min. The liquid was evaporated and the residue dissolved in H_2O and washed with ethylether, followed by extraction with H_2O-saturated *n*-butanol. The butanol layer was then evaporated to produce a saponin fraction. Each sample was dissolved in MeOH (HPLC-grade) and determination of ginsenosides was based on the method of William *et al*. (1996). The ginsenoside fractions were analyzed using a HPLC system with a *ZORBAX 300SB-C*$_{18}$ column (particle size 5 µm, 4.6 mm × 150 mm, *Agilent*, CA, USA), elution with water/acetonitrile as the mobile phase at 9:1 to 1:1 (v/v) for 40 min and a flow rate of 1.0 cm^3 min^{-1}. Ginsenosides were detected at a wavelength of 203 nm with the peak areas corresponding to ginsenosides from the samples matching retention times as authentic ginsenoside standards (Rb_1, Rb_2, Rc, Rd, Re, Rf, and Rg_1) purchased from *ChromaDex* (CA, USA). The data were integrated by comparison with an external standard calibration curve.

Hydroxyl and superoxide radical scavenging activities: The saponin fraction was precipitated by evaporation of the butanol layer, and dissolved until an approximate final concentration of 1 mg cm^{-3} in 100 % MeOH. For a chemiluminescence (CL) monitoring of the hydroxyl radical formation, a reaction mixture (1 cm^3 of 3 µmol indoxyl-β-glucuronide, IBG, 0.1 cm^3 of 1 mM $FeSO_4$, 1.6 cm^3 of 3 % H_2O_2, and 0.05 cm^3 of 10 mM EDTA) was added to a quartz round-bottom cuvette in the black-box unit of a *BPCL* ultraweak CL analyzer (*Novasis*, CA, USA) (Tsai *et al*. 2001). To assess superoxide radical formation, the reaction mixture was prepared with 1 cm^3 of 2 mM lucigenin, 1 cm^3 of a phosphate buffered saline, pH 7.4, 28 mm^3 of 1 M arginine, and 24 mm^3 of 1.4 µmol methylglyoxal (Tsai *et al*. 2003). CL was monitored at 400 nm. At a linear stage after initiation of a CL by IBG for the hydroxyl radical or lucigenin and methylglyoxal/arginine for the superoxide radical, 2 mm^3 of each extract was added to the reaction mixture. For a quantitative comparison, IC_{50} values (the concentration of a test compound needed to inhibit 50 % of a CL in the assay system) were obtained from a concentration-inhibition curve.

Statistical analysis: All parameters are expressed as the mean of at least three independent experiments. The significance of differences between control and each experimental treatment was analyzed by the Duncan's multiple range test (DMRT).

Results and discussion

Embryogenic callus induction from mature embryos: Aseptic cotyledon explants were cultured on callus induction MS medium, and amorphous embryogenic calli were produced within three weeks (Fig. 1*A*). The embryogenic callus was isolated from the cotyledon and then proliferated on the same medium by consecutive subculture. Choi *et al*. (2003) suggested that high-strength NH_4NO_3 (40 and 60 mM) suppresses embryo development from ginseng cotyledons while permitting the growth of hormone-independent amorphous embryogenic callus. In the present study, however, the embryogenic callus was induced on MS medium containing original-strength NH_4NO_3 (20 mM), and exogenously supplied auxin and cytokinin promoted the production of embryogenic callus from ginseng cotyledons (Lu *et al*. 2001).

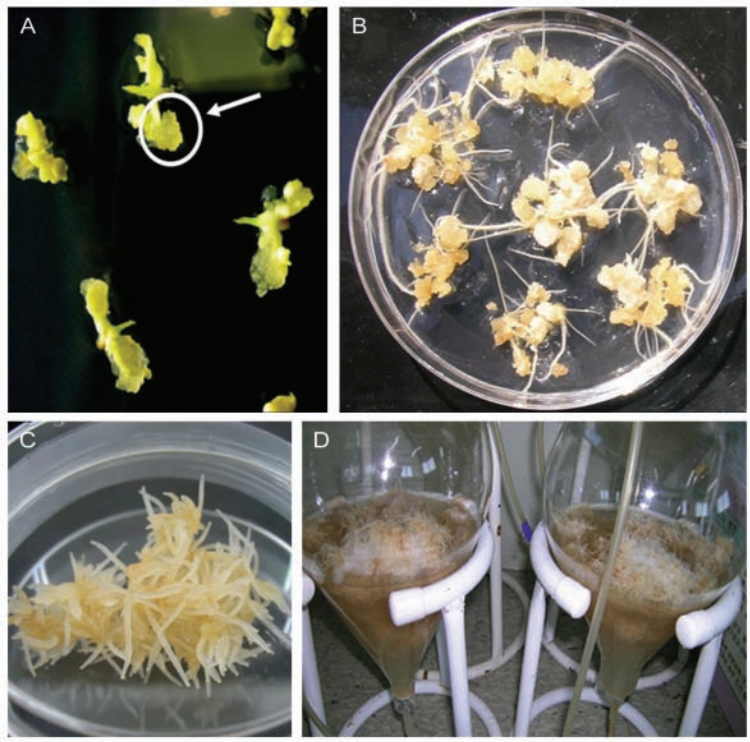

Fig. 1. Induction and proliferation of embryogenic calli and adventitious root: *A* - embryogenic calli formation on the callus induction medium, *B* - adventitious root formation on the solid adventitious root induction medium, *C* - proliferation of the adventitious root on the liquid adventitious root induction medium, *D* - mass production of the adventitious root through a 5 dm^3 air bubble bioreactor.

Table 1. Effects of IBA, NAA, 2,4-D and γ-irradiation on the frequency of adventitious root formation from embryogenic calli [%] after 4 weeks of a culture. Means ± SE of three independent experiments. Mean followed by different letters within a column are significantly different at $P < 0.05$ according to Duncan's multiple range test.

	Hormones [mg dm^{-3}]	0	0.1	0.3	1.0	3.0	5.0
Root formation [%]	IBA	0d	52.4 ± 2.8bc	42.9 ± 0.1c	66.7 ± 2.8	76.2 ± 7.3a	66.7 ± 2.8
	NAA	0c	38.1 ± 7.3ab	52.4 ± 5.5a	52.4 ± 12.0a	47.6 ± 12.0a	14.3 ± 8.3bc
	2,4-D	0a	9.5 ± 5.5a	9.5 ± 5.5a	0a	0a	0a
	Irradiation doses [Gy]	0	10	30	50	70	100
Root formation [%]		94.0 ± 0.8a	66.0 ± 3.0c	78.8 ± 1.6b	50.8 ± 3.9d	29.2 ± 2.9e	18.3 ± 2.0f
Fresh mass [mg]		479.3 ± 5.6c	503.0 ± 29.2c	713.0 ± 32.6a	595.3 ± 18.4b	459.7 ± 5.4c	444.0 ± 25.3c

Effect of plant growth regulators on adventitious root formation: To identify an appropriate type and concentration of auxin for adventitious root formation, the proliferated embryogenic callus was exposed to different concentrations (0, 0.1, 0.3, 1, 3, and 5 mg dm^{-3}) of IBA, NAA, and 2,4-D. Adventitious roots were formed within two weeks after culture initiation (Fig. 1*B*). No adventitious root was produced on a medium without auxin. The highest frequency of adventitious root formation was observed on the NH_4NO_3-free MS medium with IBA (Table 1). The adventitious root formation increased with increasing IBA concentration and maximum formation of 76.2 % was noted at 3 mg dm^{-3} IBA. However, a higher IBA concentration (5 mg dm^{-3}) decreased the frequency. In the case of NAA, the highest adventitious root formation (52.4 %) was observed at 0.3 and 1.0 mg dm^{-3}. In all the 2,4-D treatments, the adventitious root formation was less than 10 %. IBA likely plays a key role in adventitious root formation by initiating cell division and primordium structures and inducing cell dedifferentiation to form the apical meristem. A complete understanding of the molecular mechanism of IBA action during the formation of root meristem remains unknown (Berleth and Sachs 2001).

Effect of γ-irradiation on adventitious root formation: The proliferated embryogenic callus was irradiated with γ-rays (0, 10, 30, 50, 70, and 100 Gy). The frequency of adventitious root formation was decreased as the doses increased, except for the 30 Gy-treatment (Table 1). Although the frequency of adventitious root formation with non-irradiated control callus was highest (94.0 %), the maximum fresh mass was found to be higher (713.0 mg) at 30 Gy treatment compared to 479.3 mg for the control callus after 4 weeks of culture. Therefore the dose 30 Gy was considered as an adequate for the selection of mutant cell lines of ginseng. Thus, large quantities of the embryogenic callus were irradiated at 30 Gy and then transferred to an adventitious root induction medium. In a previous report, we identified that a variation spectrum by γ-irradiation at callus stages was wider than the somaclonal variation seen with tissue culture (Kim *et al*. 2003).

Selection of mutated adventitious root lines: We generated 142 ginseng adventitious root lines from the callus treated by 30 Gy of γ-rays. These root lines were characterized by having high branching and rapid growth. These root lines were designated as mutated adventitious root lines (MAR3-1 to MAR3-142) wherein the first number represents the radiation dose (1 krad = 10 Gy) and the second is the number of the root line. The control adventitious root induced from the non-irradiated callus was designated as CAR.

The CAR and MAR3 lines were transferred and allowed to proliferate in a liquid NH_4NO_3-free MS medium supplemented with 3 mg dm^{-3} IBA (Fig. 1*C*). We selected five MAR3 lines (MAR3-9, MAR3-10, MAR3-13, MAR3-26, and MAR3-109) with higher degree of branching and more rapid growth rate compared to CAR. The growth rates (final fresh mass/inoculum fresh mass) obtained after 8 weeks of culture were 4.5, 5.1, 3.1, 1.9, and 2.5-fold higher for MAR3-9, MAR3-10, MAR3-13, MAR3-26, and MAR3-109 than for CAR, respectively (Table 2). The growth rate of adventitious roots has been shown to depend on the formation of more numbers of new growth points as lateral branches and on a secondary increase in root diameter as root cells undergo cell expansion and differentiation (Rhodes *et al*. 1990). The selected MAR3-lines were transferred to a 5-dm^3 air bubble bioreactor for adventitious root mass production (Fig. 1*D*).

Table 2. Fresh mass of adventitious roots of CAR and five MAR3-lines after 4 and 8 weeks. Means ± SE of three independent experiments. Means followed by different letters within a column are significantly different at $P < 0.05$ according to Duncan's multiple range test.

Root lines	0 week	4 weeks	8 weeks
CAR	0.02	0.41 ± 0.05c	1.30 ± 0.04f
MAR3-9	0.02	0.76 ± 0.05b	5.89 ± 0.08b
MAR3-10	0.01	0.46 ± 0.04bc	3.30 ± 0.10d
MAR3-13	0.02	0.45 ± 0.03bc	3.98 ± 0.07c
MAR3-26	0.02	0.47 ± 0.04bc	2.43 ± 0.21e
MAR3-109	0.04	1.50 ± 0.13a	6.55 ± 0.18a

Ginsenoside analyses by HPLC: The content of the seven ginsenoside types of the Rb group (Rb_1, Rb_2, Rc, and Rd) and Rg group (Re, Rf, and Rg_1) from the CAR and 5 MAR3-lines were compared with the contents determined in 6-year-old *Panax ginseng* (naturally grown ginseng). Shi *et al*. (2006) reported that the seven ginsenosides in roots and root-hairs increased in the older *Panax ginseng*, whereas the results were different in leaf extracts. The ginsenoside pattern was similar in the native ginseng, CAR, and 3 MAR3-lines (MAR3-9, MAR3-10, and MAR3-109), although there were some quantitative differences. The Rd and Re ginsenosides from the native ginseng and Re ginsenosides from the CAR were undetectable by HPLC. The predominant ginsenoside found in CAR and MAR root lines was Rg_1, followed by Rb_1 or Rc (Table 3). Ginsenoside Rg_1 is a steroidal saponin of high content in ginseng and is known to relax rat aorta and pulmonary vessels *via* a release of nitric oxide. This highest accumulation of Rg_1 in the cultured CAR and MAR lines might be due to supplementation with IBA, the auxin which diminishes the activity of the enzymes responsible for a transformation of Rg_1 into Re or Rf (Bonfill *et al*. 2002).

The total ginsenoside content of the CAR was 2.5 times higher than in the native ginseng [398.16 *versus* 159.99 mg g^{-1}(d.m.)], respectively. Among the five mutant lines, the total ginsenoside content ranged from 348.93 mg g^{-1} (d.m.) in MAR3-109 to 679.51 mg g^{-1}(d.m.) in MAR3-9. The ratio of total content of Rb group ginsenosides/total content of Rg group ginsenosides (TRb/TRg) ratio is important for a pharmacological quality evaluation. In general, in the shoot the TRb/TRg ratio is less than 1, while in roots the ratio is somewhat higher (Furuya *et al*. 1986). However, the TRb/TRg ratios we determined were 0.53 and 0.57 in the native ginseng and MAR3-109 roots, respectively. The TRb/TRg ratio was higher than 1.0 in the other four MAR3-lines and CAR. The TRb/TRg ratio of the MAR3-13 and MAR3-26 was the highest (1.64 and 1.61, respectively) because of the relatively low TRg compared to the other MAR3-lines. This result is in agreement with suggested promotion of the synthesis of the Rb group ginsenosides by IBA (Bonfill *et al*. 2002). In addition, it must also be considered that genes encoding enzymes that induce synthesis of the Rb group ginsenosides might be mutated, and therefore these genes might lead to an increase of the Rb group ginsenoside synthesis.

The HPLC analysis of the MAR3-13 and MAR3-26 demonstrated different ginsenoside profiles compared to other root lines and the native ginseng. It is of interest that three unidentified ginsenoside candidates were observed which we designated as Gm_1, Gm_2, and Gm_3 (Fig. 2). The Gm_2 ginsenoside was detected at the highest level in these two lines. The peak areas of Gm_2 in the MAR3-13 and MAR3-26 were 46 and 61 % higher than that of each Rg_1 ginsenoside, respectively. Compared to Rg_1, the peak areas of Gm_1 and Gm_3 were about 65 and 53 %, and 64 and 57 % for the MAR3-13 and MAR3-26, respectively. The enhanced accumulation of the three Gm compounds in these MAR3-13 and MAR3-26 lines suggests a significant effect of γ-irradiation on the ginsenoside biosynthesis. Relatively low content of Rg_1 ginsenoside in two MAR3-lines seemed to be related to alterations of key enzymes for the biosynthesis of the protopanaxadiol and protopanaxatriol, which are derived from dammarenediol by hydroxylations, and/or a transformation of Rg_1 into Re or Rf. The individual ginsenoside profiles were greatly influenced by the difference of enzyme activity involved in the ginsenoside pathway (Bonefill *et al*. 2002), or the cultured root line phenotype and the elicitation conditions (Palazón *et al*. 2003).

Table 3. Content of ginsenosides [mg g^{-1}(d.m.)] in native ginseng, CAR, and five MAR3-lines (TRb = $Rb_1 + Rb_2$ + Rc + Rd, TRg = Re + Rf + Rg_1). Each value represents the mean of three replicates. Means followed by different letters within a column are significantly different at $P < 0.05$ according to Duncan's multiple range test.

Root lines	Rb_1	Rb_2	Rc	Rd	TRb	Re	Rf	Rg_1	TRg	TRb/TRg
Native ginseng	28.30e	4.50e	22.80e	-	55.60	-	11.65c	92.74e	104.39	0.53
CAR	61.86d	53.36c	80.73b	20.05c	216.00	-	13.21c	168.95c	182.16	1.19
MAR3-9	101.27c	104.72a	125.88a	25.66b	357.53	14.81b	24.62b	282.55a	321.98	1.11
MAR3-10	123.73b	76.76b	81.90b	7.18d	289.57	14.59b	7.18d	194.86b	216.63	1.34
MAR3-13	122.58b	83.76b	47.77d	27.89b	282.00	31.85a	12.60c	127.86d	172.31	1.64
MAR3-26	145.39a	39.23d	72.17c	38.92a	295.71	31.78a	30.58a	121.11d	183.47	1.61
MAR3-109	21.33e	10.09e	87.36d	7.31d	126.09	5.03c	25.01b	192.80b	222.84	0.57

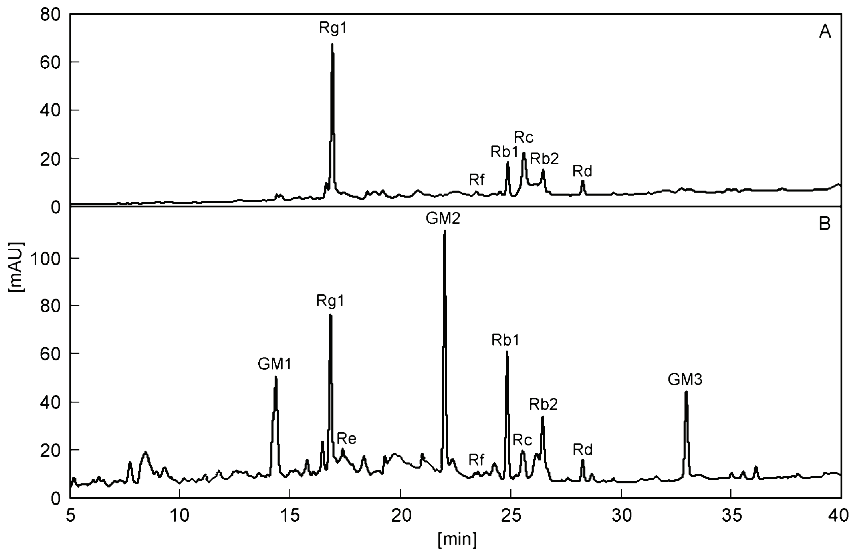

Fig. 2. HPLC chromatograms of ginseng extracts in the CAR (*A*) and MAR3-13 (*B*). Seven ginsenosides, including Rb_1, Rb_2, Rc, and Rd of the Rb group, Re, Rf, and Rg_1 of the Rg group, and three unidentified ginsenoside candidates, including Gm_1, Gm_2, and Gm_3, were marked upon each peak area.

In addition, relatively low Rb_2 and Rc contents may also contribute to the biosynthesis of the three Gm compounds *via* a difference in preferences for the ginsenoside biosynthetic pathway between the two MAR3-lines and other root lines. For the identification of the three Gm compounds and elucidation of their structure by ^{1}H and ^{13}C NMR, FT-IR spectrometers, and EI mass station, the MAR lines were proliferated in bioreactor. In addition, enzymes involved in ginsenoside biosynthesis are going to be investigated.

Table 4. IC_{50} [μg cm^{-3}] of superoxide and hydroxyl radical scavenging activities on the native ginseng, CAR, and 5 MAR3-lines using the ultraweak CL analyzer. Each value is sample concentration for 50 % inhibition of supplemented radical. Radical scavenging activities (fold) of cultured CAR and 5 MAR3-lines (AR) compared with that of native ginseng (NG). Means ± SE of three independent experiments. The means followed by different letters within a column are significantly different at $P < 0.05$ according to Duncan's multiple range test.

Root lines	Hydroxyl	NG/AR	Superoxide	NG/AR
NG	3.67 ± 0.15a[c]	1.00	9.05 ± 0.05a	1.00
CAR	3.10 ± 0.08b	1.19	5.70 ± 0.03b	1.59
MAR3-9	2.30 ± 0.13d	1.60	5.06 ± 0.06c	1.79
MAR3-10	3.23 ± 0.07b	1.14	3.42 ± 0.12e	2.65
MAR3-13	1.01 ± 0.03e	3.65	1.11 ± 0.05g	8.18
MAR3-26	2.67 ± 0.07c	1.38	1.75 ± 0.05f	5.17
MAR3-109	2.68 ± 0.05c	1.37	3.71 ± 0.10d	2.44

Hydroxyl and superoxide radical scavenging activities: Recently, it was reported that ginsenoside accumulation and the enzymatic and non-enzymatic antioxidants in suspension culture of *Panax ginseng* roots are induced by methyl jasmonate (MA) and salicylic acid (SA) (Ali *et al*. 2006) or by oxidative stress (Ali *et al*. 2005). In this study, we investigated the hydroxyl and superoxide radical scavenging activities of the root lines using a ultraweak CL analyzer with a high-sensitivity detector. The hydroxyl scavenging ability of the CAR (IC_{50} value 3.08 μg cm^{-3}) was 19 % higher than that of native ginseng (3.67 μg cm^{-3}) (Table 4). The highest hydroxyl scavenging abilities was observed in the MAR3-13 (1.01 μg cm^{-3}), followed by MAR3-9, MAR3-26, MAR3-109 and MAR3-10.

The superoxide scavenging ability of the CAR was 59 % higher than that of the native ginseng (Table 4). All MAR3-lines showed a higher superoxide scavenging ability compared to the CAR. This result demonstrates that the three compounds, Gm_1, Gm_2, and Gm_3, of the MAR3-13 and MAR3-26 lines might increase superoxide scavenging ability.

As it is known, in root cultures, the ginsenoside yield depends not only on the accumulation of the commercially useful compounds but also on their biomass production capability. In this study, we selected the MAR3-lines with a significantly increased biomass productivity and ginsenosides content compared to the CAR and with significant variation in ginsenosides pattern. We anticipate that the selected MAR3-lines will be also useful in studies related to the biosynthetic pathway of the ginsenosides in *Panax ginseng*.

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