Journal of Plant Biology, February 2006, 49(1) : 26-33

Induction of Adventitious Roots and Analysis of Ginsenoside Content and the Genes Involved in Triterpene Biosynthesis in *Panax ginseng*

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Adventitious roots were produced directly from root segments of *Panax ginseng* seedlings when cultured on an MS solid medium containing 3.0 mg L^{-1} IBA. Omitting NH_4NO_3 from this medium greatly enhanced both the frequency of adventitious root formation and the number of roots per explants. This frequency declined markedly with the age of the root, but could be increased through repeated sub-culturing events. A two-step procedure that included NH_4NO_3 free media for the first two weeks of culture, followed by transfer onto media containing NH_4NO_3 for another four weeks, greatly improved total fresh weights of these adventitious roots compared with a method of continuous culture over six weeks in media that always contained NH_4NO_3. Expression of the genes involved in triterpene biosynthesis was analyzed by RT-PCR. Ginsenoside contents were enhanced by the omission of NH_4NO_3 and were also greatly increased by treatment with methyl jasmonate.

Keywords: adventitious roots, ginsenoside, NH_4NO_3, *Panax*, RT-PCR, triterpene

Panax ginseng is a perennial herbaceous plant in the Araliaceae family. Its roots have long served important medicinal purposes in promoting the quality of life (Ellis and Reddy, 2002; Coleman et al., 2003). In laboratory and clinical trials, its most notable features have been modulation of the immune system, and activities against stress effects, cancer, and diabetes (Vogler et al., 1999; Dey et al., 2003; Kiefer and Pantuso, 2003; Yun, 2003). Its representative secondary compounds are ginsenosides, or saponins, which are dammarane-type triterpene glycosides that are constituted with tetracyclic aglycon in *Panax*. In that genus, more than 25 different ginsenosides are produced naturally; their contents and constitutions differ by species (Shibata, 2001).

Ginseng root is expensive because of difficulty with its cultivation. For wild ginseng, the cost is about 20- to 100-fold higher. Plant tissue culture methods have been explored as potentially efficient alternatives for the mass production of ginseng cells and tissues. Cells derived from pilot-plant cultures have been applied commercially to various foods and cosmetics in Japan, e.g., by the Nitto Denko Company (Ushiyama, 1991). Recently, an *in vitro* adventitious root culture system has been reported (Kevers et al., 1999). Such a system has some advantages because the adventitious roots contain the same saponin (ginsenoside) as those of the native roots (Choi et al., 2000; Yu et al., 2002), and can be produced in a large-scale bioreactor (Yu et al., 2002).

The objective of our study was to establish an adventitious root culture system for wild-cultivated and true-wild *P. ginseng*. We investigated the influence of NH_4NO_3 on the induction and elongation of adventitious roots, in particular its effect plus that of methyl jasmonate (MeJa) on ginsenoside accumulation as well as the expression of genes related to triterpene biosynthesis.

MATERIALS AND METHODS

Plant Materials

True-wild ginseng (*P. ginseng* C.A. Meyer) plants were collected from Hambaek Mountain at Jeongseon-Kun, in the Kangwon province, Korea. By counting the leaf abscission scars on their short stems, we were able to estimate their age as at least 50 years old. In addition, we gathered seeds and plants of *P. ginseng* that were being cultivated in the wild on a

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mountain near Samchuck-Kun, also in Kangwon province. Before germination, dehisced seeds were moisture-chilled for 6 months, then immersed in 70% ethanol for one min and 1% sodium hypochlorite for 10 min. Their zygotic embryos were removed, then cultured on a 1/2-strength MS medium (Murashige and Skoog, 1962) containing 1% sucrose, and solidified with 0.27% gelrite. After two weeks of culture, their roots were aseptically cut into 1.0-cm segments for culturing. Roots from wild-cultivated and true-wild ginseng plants also were surface-sterilized by the above protocol. Fine roots (<1.5 mm diam.) were cut into 1-cm segments.

Influence of NH_4NO_3 on Adventitious Root Induction

Root segments of two-week-old seedlings were cultured on one of three media types: MS, 1/2 MS, or 1/2 MS lacking NH_4NO_3. Each contained 3.0 mg L^{-1} IBA and 3% (w/v) sucrose, and was solidified with 0.27% gelrite, adjusted to pH 5.8, then autoclaved at 120°C for 15 min. About 30 root segments per Petri dish were cultured for 5 weeks at 22 ± 1°C under darkness. This experiment was repeated three times. Photographs were taken at 2 and 5 weeks, and after 5 weeks of culture, the frequency, number, and lengths of adventitious roots were recorded.

Adventitious Root Induction from Wild-Cultivated and True-Wild Ginseng Roots

The fine-root segments prepared from wild-cultivated (1, 5, or 15 years old) and true-wild ginseng (at least 50 years old) were cultured on a 1/2 MS medium that lacked NH_4NO_3 but contained 3.0 mg L^{-1} IBA and 3% (w/v) sucrose, and was solidified by 0.27% gelrite. After being adjusted to pH 5.8 the medium was autoclaved at 120°C for 15 min. About 30 root segments per Petri dish were cultured for 5 weeks under darkness at 22 ± 1°C. This experiment was repeated three times. After 5 weeks, the frequency of adventitious root formation and the number of roots produced per explant were recorded. Induced adventitious root tips were excised from the maternal explants and sub-cultured on the same type of medium as that for the initial root induction. Sub-culturing was performed 7 times, at 5-week intervals. The frequency of adventitious root formation and the number of roots per explant were recorded after the 1st, 3rd, 5th, and 7th sub-culturing events.

Shake-Flask Cultures and MeJa Treatment

Adventitious roots from true-wild ginseng roots that proliferated on the 1/2 MS solid medium lacking NH_4NO_3 were excised in 10-mm lengths, and 300 mg of fresh tissue was transferred to 100-ml Erlenmeyer flasks containing 30 mL of one of three media types: MS, 1/2 MS, or 1/2 MS liquid medium devoid of NH_4NO_3. Each medium comprised 3.0 mg L^{-1} IBA and 3% sucrose. For the two-step culture procedure, adventitious root segments (300 mg) were cultured on a 1/2-strength MS medium lacking NH_4NO_3 for the first 2 weeks, then transferred to a 1/2 MS medium with NH_4NO_3 for a further 4 weeks. The increase in fresh weights for adventitious roots was recorded weekly during the 6-week culture period. A separate experiment involved the influence of elicitors on ginsenoside accumulation and the expression of genes involved in triterpene biosynthesis. Here, adventitious root segments were cultured in a 1/2 MS liquid medium with 10 mM MeJa. Flasks were agitated on a rotary shaker at 100 rpm. Each treatment consisted of three flasks, and the experiment was repeated three times. The culture room was maintained at 22 ± 1°C under 20 μmol m^{-2} s^{-1} from white fluorescent lamps.

Ginsenoside Analysis by HPLC

Ginsenosides were extracted as described by Ando et al. (1971). One gram of milled powder from freeze-dried adventitious roots (sampled after 4 weeks of culture) was soaked in 80% MeOH at 60°C. After the liquid was evaporated, the residue was dissolved in H_2O and washed twice, followed by extraction with H_2O-saturated n-butanol. The butanol layer was then evaporated to produce the saponin fraction. Each sample was dissolved in EtOH, then filtrated with a SepPak C-18 Cartridge (Waters, USA). HPLC separation was conducted on a NovaPak C18 column (4 μM, 3.9 X 150 mm; Waters), applying the following gradient system: 0 min, 100% acetonitrile; 10 min, 75% acetonitrile and 25% water; 25 min, 67% acetonitrile and 37% water. The flow rate of the mobile phase was 1.2 mL min^{-1}, and the ginsenosides were monitored at 202 nm. Each ginsenoside was compared with its authentic ginsenoside (Chroma-Dex, USA). Quantitative analysis was performed by the one-point curve method using the authentic ginsenosides as external standards.

RT-PCR Analysis

Total RNAs were isolated from adventitious roots cultured either for 4 weeks on different media (MS, 1/2 MS, or 1/2 MS lacking NH_4NO_3) or for 24 h on a 1/2 MS medium supplemented with 10 μM MeJa. They were then reverse-transcribed by the ImProm-II Reverse Transcription System (Promega, USA). The first-strand cDNAs were used as template for reverse transcription-PCR analysis, performed as follows: 96°C for 5 min; then 30 cycles of 96°C for 30 s, 60°C for 30 s, and 72°C for 1 min; with a final 10-min extension at 72°C. Primers included 5′-ATGGGAAGTTTGGGGGCAATTCT-3′ 5′-GTTCTCACTGTTTGTTCAGTAGTAGGTT-3′ for *P. ginseng PgSS1* (squalene synthase, AB115496), 5′-AGCAGCAGTTGACAAAGG-3′ 5′-GCCACATTCGTTTTGGTGAAGG-3′ for *P. ginseng PgSE* (squalene epoxidase, AB003516), 5′-TCATCAGATGGCTCATGGTACG-3′ 5′-TCTCCTCCTGTGGGAAATCACC-3′ for *P. ginseng PNX* (cycloartenol synthase, AB009029), 5′-TATCCTGGACACCGAAAGAAGG-3′ 5′-CTCCACTTATTTCCTGTTGGGG-3′ for *P. ginseng PNY* (β-amyrin synthase, AB025345), 5′-GTGGATTTTCTAATAAAATCGCAACGCAG-3′ 5′-TTTCATTTGAGTATTGGCAGGCCG-3′ for *P. ginseng PNY2* (β-amyrin synthase, AB014507), and 5′-ATGTGCAAGCTGAAGGTTGCTCAAGGA-3′ 5′-TTAAATTTTGAGCTGCTGGTGCTTAGGC-3′ for *P. ginseng PgOSC1* (putative β-amyrin synthase, AB122080). RT-PCR analysis of β-actin was used as the control to check for RNA integrity and accuracy of loading.

RESULTS AND DISCUSSION

Direct Adventitious Root Induction

In a preliminary test, we found that 3.0 mg L^{-1} IBA was the most suitable auxin for inducing adventitious root formation from segments of true-wild *P. ginseng*. Thus we selected this particular concentration for further induction experiments. Roots from germinated seedlings of wild-cultivated ginseng were cut into 10-

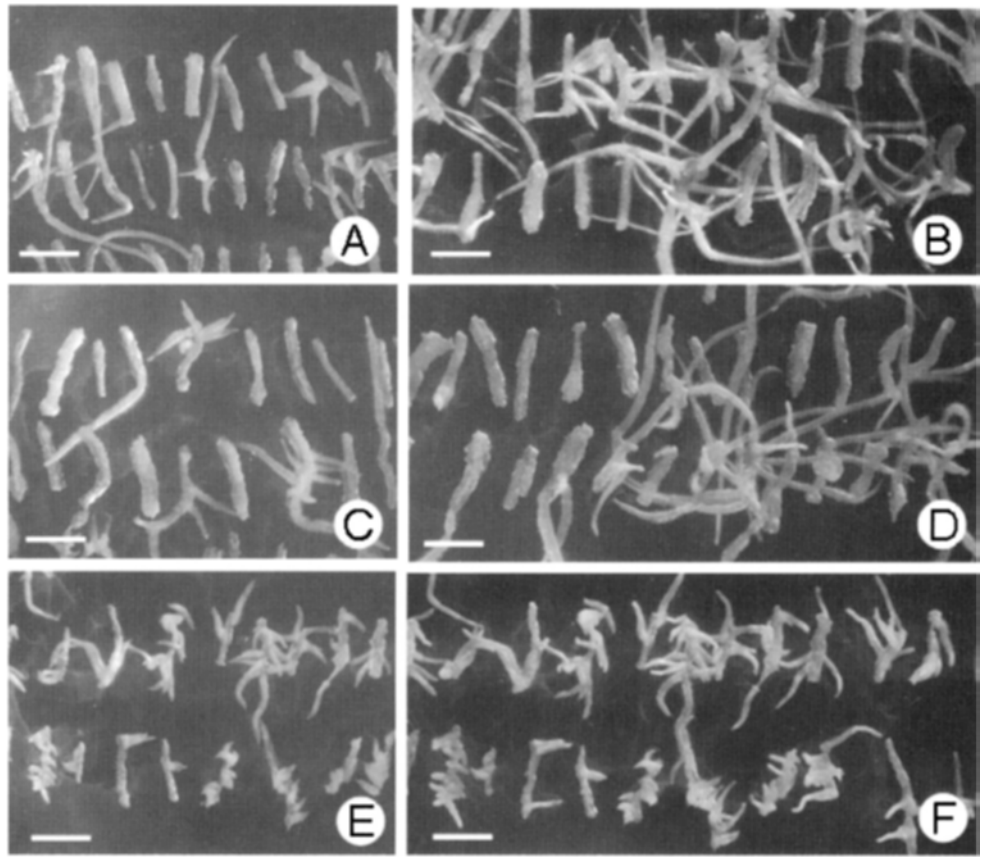

Figure 1. Direct adventitious root formation from segments of 2-week-old ginseng seedling roots on various media. **A-B,** Induction on full-strength MS medium after 2 **(A)** and 5 weeks of culture **(B); C-D**, Induction on half-strength MS medium after 2 **(C)** and 5 weeks of culture **(D); E-F,** Induction on half-strength MS medium devoid of NH_4NO_3 after 2 **(E)** and 5 weeks of culture **(F)**. Bars = 10 mm.

Table 1. Influence of media strength and NH_4NO_3 on the induction and growth of adventitious roots in root segments of *P. ginseng* seedlings after five weeks of culture.

Medium	Adventitious root formation (%)	No. of roots/explant	Length of root (cm)	Total fresh weight (mg)
MS	29 ± 3.2	7.23 ± 0.83	1.16 ± 0.02	1196 ± 142.2
1/2 MS	25 ± 2.6	10.76 ± 0.9	1.22 ± 0.13	1026 ± 45.2
1/2 MS without $NH4^+$	91 ± 6.5	14.15 ± 2.3	0.48 ± 0.32	490 ± 30.9

Data represent mean ± SE from three independent experiments.

mm segments and cultured on three different IBA-supplemented solid media (MS, 1/2 MS, or 1/2 MS lacking NH_4NO_3). Most of the adventitious roots formed directly on the segment surfaces after two weeks of culture (Fig. 1A, 1C, 1E), followed by root elongation (Fig. 1B, 1D, 1F) until after 5 weeks of culture. Omitting the NH_4NO_3 from the media greatly enhanced the frequency of adventitious root formation and the number of roots per explant compared with the results from using full- or half-strength MS media that did contain NH_4NO_3 (Fig. 1E, 1F; Table 1). Nevertheless, further elongation of post-induced adventitious roots was very slow on the 1/2 MS medium lacking NH_4NO_3 compared with roots placed on the MS or 1/2 MS medium with NH_4NO_3. The longest adventitious roots developed on the 1/2 MS medium with NH_4NO_3. These results indicate that, although lowering the level or entirely omitting NH_4NO_3 may be effective for inducing direct formation of adventitious roots in *P. ginseng*, its presence eventually is necessary for their further growth and elongation.

Growth of *Lithospermum erythrorhizon* plants is strongly influenced by the proportion of NH_4^+/NO_3^- in the medium (Shimomura et al., 1991). Likewise, in carrot cell suspension cultures, a high level of reduced NH_4^+ fosters somatic embryogenesis from embryogenic cells whereas its omission induces adventitious root formation from cell clumps (Halperin, 1966). Similar alternative development of somatic embryos and adventitious roots that depends on the concentration of NH_4NO_3 has been observed in cotyledon cultures of *Panax ginseng* (Choi and Soh, 1997). Furthermore, in that species, a 1:1 proportion of NH_4^+ to NO_3^- is optimal for increasing the fresh weights of those adventitious roots (Lian et al., 2002).

Adventitious Root Induction from Wild-Cultivated Ginseng and True-Wild Ginseng

Root segments of wild-cultivated ginseng (1, 5 and 15 years old; Fig. 2A-C) and true-wild ginseng (supposedly at least 50 years old; Fig. 2D) were cultured on a 1/2 MS medium lacking NH_4NO_3. Their frequency of root formation and the number of roots produced per segment markedly declined with the age of the roots (Table 2). In the older, true-wild ginseng, only about 3% of the root explants produced adventitious roots, with an average of <1 root being counted from each explant. However, when the tips of those induced true-wild adventitious roots were isolated and sub-cultured at 5-week intervals, the frequency of adventitious root formation increased rap-

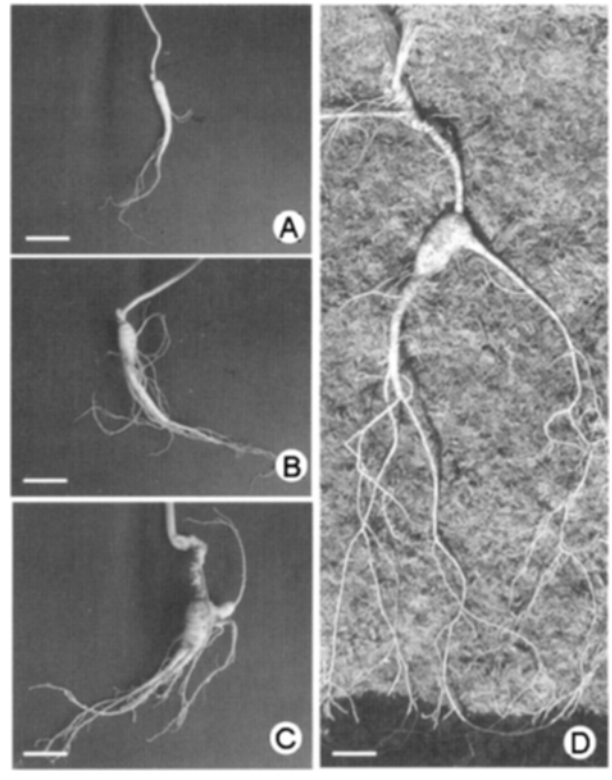

Figure 2. A-C, Wild-cultivated ginseng (**A** = one year old, **B** = 5 years old, **C** = 15 years old); **D,** True-wild ginseng (at least 50 years old). Bars, **A-C** = 10 mm; D = 20 mm.

Table 2. Age-dependent frequency of adventitious root formation from wild-cultivated and true wild ginseng roots after 5 weeks of culture.

Type of root	Age of root (years)	Adventitious root formation (%)	No. of roots/ explant
Wild-cultivated	1	52.5 ± 5.3	4.2 ± 0.31
	5	23.2 ± 2.7	3.6 ± 0.12
	15	12.7 ± 3.5	1.5 ± 0.03
True-wild	50	2.6 ± 1.9	0.4 ± 0.02

Data represent mean ± SE from three independent experiments.

Table 3. Increase in adventitious root formation after repetitive sub-culturing (at 5-week intervals) of adventitious roots in true-wild *P. ginseng* after 5 weeks of culture.

Subculture period (weeks)	Frequency of adventitious root formation (%)	No. of adventitious roots/explant
1	2.6 ± 1.9	0.4 ± 0.02
3	56.4 ± 3.2	2.3 ± 0.52
5	89.2 ± 11.1	4.7 ± 0.95
7	100.0	7.3 ± 2.1

Data represent mean ± SE from three independent experiments.

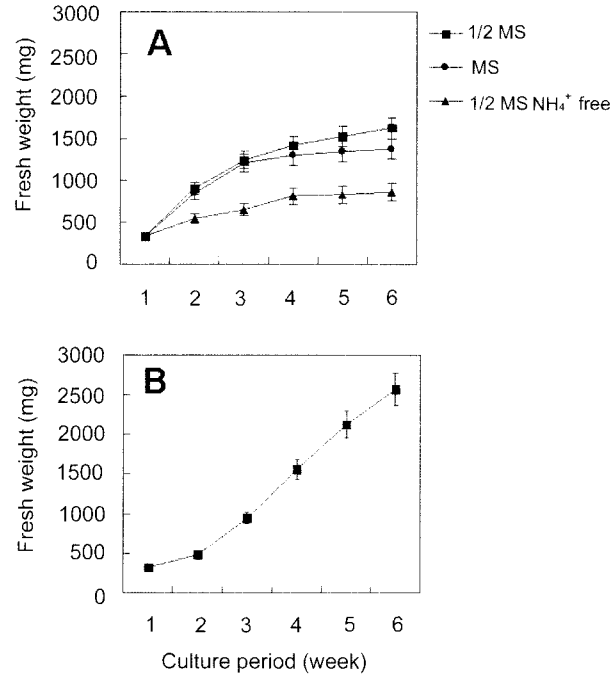

Figure 3. Increase in fresh weights of *P. ginseng* adventitious roots during 6 weeks of shake-flask culturing. **A,** Growth of adventitious roots in continuous culture on MS, 1/2 MS, or 1/2 MS medium lacking NH_4NO_3; **B,** Growth of adventitious roots via two-step culture method (NH_4NO_3-free medium for first two weeks, then in medium supplemented with NH_4NO_3 for further 4 weeks).

idly with each sub-culturing event (Table 3). In fact, when sub-culturing was done seven times, 100% of the root tips produced adventitious roots, with an average of seven roots per explant (Table 3). This demonstrates that losing the capacity to induce adventitious roots is closely associated with plant age and maturity. Roulund (1973, 1975) reported that the pattern of growth and development for vegetatively propagated plants differs significantly from that of seedling-derived plants, and that the frequency of vegetative propagation by stem cuttings declines with accumulated age and maturity. This phenomenon is called cyclophysis (Olesen, 1978). Such an increase in adventitious root formation through consecutive sub-culturing may result from the attainment of rejuvenility. Under micropropagation, repeated sub-culturing of the apical meristem causes a reversion to the juvenile stage from the mature phase (Mullins and Sampet, 1979). Furthermore, slices of wild ginseng roots cultured on a medium with 2,4-D develop adventitious roots when the induced calli are transferred to a medium containing NAA or IBA (Choi et al., 2000; Yu et al., 2002). Therefore, direct adventitious root induction from segments is a suitable protocol for *P. ginseng*.

Two-Step Shake-Flask Cultures

Liquid culture is advantageous for large-scale operations. Here, excised adventitious root segments (300 mg fresh weight) originated from true-wild ginseng were formed on a 1/2-strength MS solid medium in the absence of NH_4NO_3 (Fig. 3A). They were then inoculated into flasks containing MS, 1/2 MS, or 1/2 MS media that each contained 3 mg L^{-1} IBA and 3% sucrose, but that lacked NH_4NO_3 in the third type. Growth was assessed at one-week intervals during 6 weeks of culture. Total fresh weights were highest in the 1/2 MS medium with NH_4NO_3 and lowest in the 1/2 MS medium lacking NH_4NO_3 (Fig. 3A).

Because the induction and elongation of adventitious roots require different concentrations of NH_4NO_3, we designed a two-step culture system that changed the concentration of NH_4NO_3 over time (Fig. 3B). Here, the root segments were first cultured for 2 weeks in a 1/2 MS medium lacking NH_4NO_3, then transferred to a 1/2 MS medium with the origi-

nal concentration of NH_4NO_3 for another four weeks of culture. This two-step method doubled the fresh weights of the adventitious roots compared with those under continuous culture on MS, 1/2 MS and 1/2 MS media that had contained NH_4NO_3 for the entire period.

Influence of NH_4NO_3 on the Expression of Genes Involved in Triterpene Synthesis and Ginsenoside Accumulation

We used RT-PCR to examine the expression of genes related to triterpene (ginsenoside) biosynthesis (i.e., *PgSS1, SE, PNX, PNY, PNY2,* and *PgOSC1*) in adventitious roots cultured on different types of media (MS, 1/2 MS, or 1/2 MS lacking NH_4NO_3). The absence of NH_4NO_3 resulted in enhanced expression of all those genes (Fig. 4A) compared with their response on the normal MS and 1/2 MS media. In contrast, the accumulation of *PNX* (cycloartenol synthase) mRNAs regulating phytosterol biosynthesis remained relatively constant regardless of the presence of NH_4NO_3 (Fig. 4A). Depriving the cultures of NH_4NO_3 stimulated the accumulation of ginsenosides by up to about 3-fold (Fig. 4B). Enhancement of seven ginsenosides (Rb_1, Rb_2, Rc, Rd, Re, Rf, and Rg_1) occurred evenly in adventitious roots cultured on the 1/2 MS medium lacking NH_4NO_3. This demonstrates that the ammonium ion in the culture medium affects the genes regulating triterpene biosynthesis and ginsenoside accumulation. In cell cultures of *Panax quinquefolium*, production of ginsenosides is maximized when nitrate is the sole nitrogen source (Zhong and Wang, 1998). Likewise, in *Lithospermum erythrorhizon*, shikonin synthesis in cell cultures is inhibited by ammonium ions in the culture media (Tabata and Fujita, 1985).

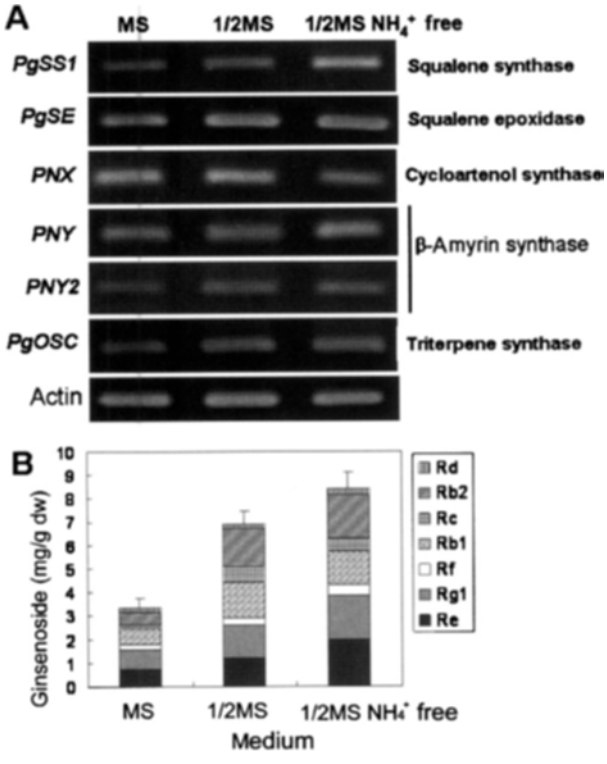

Figure 4. Analysis of ginsenoside accumulation and genes involved with ginsenoside biosynthesis in adventitious roots on MS, 1/2 MS, or 1/2 MS medium lacking NH_4NO_3, after 4 weeks of culture. **A,** Expression of *PgSS1, PgSE, PNX, PNY, PNY2,* and *PgOSC1* determined by RT-PCR; **B,** Ginsenoside content in adventitious roots.

Influence of Methyl Jasmonate on Expression of Genes Involved in Triterpene Synthesis and Ginsenoside Accumulation

Exogenous application of the elicitor MeJa stimulates the biosynthesis of many secondary metabolites (Gundlach et al., 1992). MeJa also enhances the ginsenoside content in cultured cells (Lu et al., 2001) and the adventitious roots of *P. ginseng* (Yu et al., 2002). Furthermore, the genes involved in triterpene biosynthesis are up-regulated by MeJa (Lee et al., 2004; Choi et al., 2005). Chung et al. (2003) have used DNA microarrays to show that two gene groups (metabolic genes and those that encode heat shock genes) are responsible for ginsenoside production in hairy roots of ginseng.

Expression of *PgSS1, PgSE, PNX, PNY, PNY2,* and *PgOSC1* in our adventitious roots was also examined by RT-PCR after 24 h of treatment with 10 μM MeJa. This treatment resulted in obvious accumulations of *PgSS1, PgSE,* and all three triterpene synthase *(PNY, PNY2, and PgOSC1)* mRNA in adventitious roots compared with the response by the control (Fig. 5A). In contrast, the accumulation of *CAS* (cycloartenol synthase) mRNAs was relatively constant (Fig. 5A). After 5 weeks of culture, MeJa also markedly stimulated the accumulation of ginsenosides, up to about 4-fold higher compared with the control (Fig. 5B). Production of the ginsenosides Rb_1, Rb_2, Rc, Re, Rf, and Rg_1 was also improved by MeJa treatment, while that of the ginsenoside Re remained constant. This suggests that the MeJa treatment coordinately up-regulates the expression of genes involved in triterpene biosynthesis; the exception is the *CAS* gene for phytosterol synthesis. Similarly enhanced expres-

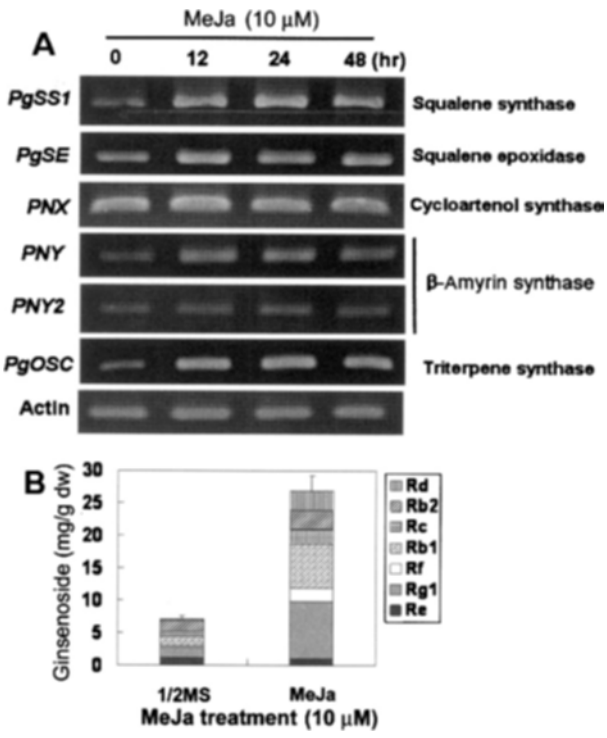

Figure 5. Ginsenoside accumulation, and analysis of genes involved with ginsenoside biosynthesis in adventitious roots. **A**, Expression of *PgSS1, PgSE, PNX, PNY, PNY2,* and *PgOSC1* after 24 h of incubation with 10 μM MeJA, determined by RT-PCR; **B,** Ginsenoside content in adventitious roots after 4 weeks of culturing on medium supplemented with 10 μM MeJA.

sion with triterpene biosynthesis has been observed in *Glycyrrhiza glabra* (Hayashi et al., 2003) and *Medicago truncatula* (Suzuki et al., 2002). In fact, this enhancement of ginsenoside accumulation and gene expression by MeJa is greater than the effect gained by eliminating NH_4NO_3 from the culture medium.

In conclusion, we have developed a new system that omits NH_4NO_3 from the culture medium in order to induce adventitious root induction in wild-cultivated and true-wild ginseng. Here, media components, particularly NH_4NO_3, affect the accumulation of ginsenoside and the expression of genes related to triterpene biosynthesis. However, this influence is less than the one gained when methyl jasmonate is used as an elicitor.

ACKNOWLEDGEMENT

This work was supported by a grant from the Korea Research Foundation (KRF-2004-042-F00007).

Received October 12, 2005; accepted November 3, 2005.

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