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Ginsenoside Rb_1 in asymmetric somatic hybrid calli of *Daucus carota* with *Panax quinquefolius*

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Abstract American ginseng (*Panax quinquefolius* L.) is one of the most valuable herbs in the world. Its major active components are ginsenosides. In order to produce ginsenoside heterogeneously, somatic hybridization, a novel approach for genetic introgression, was employed in this study. Protoplasts derived from respective calli of carrot (*Daucus carota* var. *sativus* Hoffm.) and American ginseng (*P. quinquefolius* L.) were used as the fusion partners. Hybrid calli derived from single cell lines containing chromatin of American ginseng were confirmed by the analyses of isozyme, Random amplified polymorphic DNA (RAPD) and genomic in situ hybridization (GISH). High performance liquid chromatography (HPLC) results showed that the ginseng monomer Rb_1 was synthesized in seven of the hybrid calli identified as well as in the parent American ginseng calli but not in the parent carrot calli. Results indicated that hybrid introgression lines could produce ginsenoside Rb_1 and the ginsenoside Rb_1 biosynthesis pathway has been introgressed into carrot cells via somatic hybridization. From the point of biosafety view concerning the consumer acceptance, the potential predominance to produce ginsenosides with somatic hybridization other than with genetic transformation is discussed.

Keywords American ginseng · Carrot · Somatic hybridization · Introgression cell line · Ginsenoside Rb_1

Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
6-BA	6-Benzylaminopurine
GISH	Genomic in situ hybridization
HPLC	High performance liquid chromatography
IAA	Indoleacetic acid
PAGE	Polyacrylamide gel electrophoresis
PEG	Polyethylene glycol
RAPD	Random amplified polymorphic DNA

Introduction

American ginseng (*P. quinquefolius* L., $2n = 48$), which was first cultivated in Canada and the eastern part of the United States, has been one of the most important herbal plants in the world. The most active ingredients of American ginseng are its secondary metabolites of triterpene saponins, which are also referred to as ginsenosides and generated from the isoprenoid biosynthetic pathway. Among more than 30 different ginsenosides reported in ginseng, the major ginsenosides of American ginseng are Rb_1, Rb_2, Re and Rg_1 (Li et al. 1996). The pharmacological effects of ginsenosides have made American ginseng one

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of the most widely used herbs. During over 300 years of over-harvesting, deforestation, and decreased land development, wild American ginseng populations have become typically smaller and have been listed in Appendix II of the convention on international trade in endangered species (Schlag and McIntosh 2006). Several cultivated American ginseng populations have also been developed during the recent 100 years (Schlag and McIntosh 2006). However, even cultivations still require an average of 4 years in field and their seeds must be stratified for an additional 12–18 months before germination (Zhou and Brown 2006). The difficulties in interspecies cross breeding are also major obstacles for the plant improvement using conventional ginseng breeding approaches (Proctor and Bailey 1987).

The current supply of ginseng depends mainly on field cultivation which is a slow growing and laborious process, and sometimes the quality of ginsenosides was affected by the contamination of pesticides and heavy metals (Ali et al. 2006). On the other hand, the ginsenosides outputs of both the wild and the cultivated ginseng can also be easily affected by many factors, such as populations (Lim et al. 2005; Schlag and McIntosh 2006), root age (Court et al. 1996; Lim et al. 2005), environmental conditions (Fournier et al. 2003) and locations (Jackson et al. 2003).

The exigent cultivating conditions as well as the medical and commercial value of American ginseng, therefore, have made its bioengineering a subject for investigation. It is known that in vitro culture has no seasonal or regional restrictions, so this technique has been utilized for the clonal propagation and efficient productions of plant metabolites (Davey et al. 2005). The in vitro culture system for American ginseng has been established and optimized (Teng et al. 2002; Zhou and Brown 2006). However, the growth period from regenerated plants to mature plants suitable for extraction of ginsenosides still requires 4–6 years (Kim et al. 2004).

Plant cell culture was thus considered as one of the methods of ginsenoside production. *Panax ginseng* C.A. Meyer, a very close relative species of American ginseng, has been well studied by many reports (Wu and Zhong 1999). Until now, only embryonic tissues (Asaka et al. 1993), adventitious (Yu et al. 2000a) and the hairy root (Yu et al. 2000b) of *P. ginseng* have been used in the bioreactor research for ginsenoside production. But there are very limited studies related to ginsenoside accumulation by the tissue culture of *P. quinquefolium* (Jian et al. 1998).

Plant somatic hybridization has been suggested as a means of bypassing the sexual incompatibility to produce hybrids. In addition, it enables the transfer of nuclear as well as cytoplasmic genes. Many intra- and inter-specific, intergeneric, intertribal, and even inter-familial somatic hybrid plants have been obtained (Dudits et al. 1987; Kisaka et al. 1994; Wang et al. 2005; Zhou et al. 2006). One of the advantages of somatic hybridization is that novel genetic combinations—especially the traits controlled by multiple genes—could be produced. Male sterility (Chuong et al. 1988), cold tolerance (Vazquez-Thello et al. 1996) and metal accumulation (Brewer et al. 1999) have been studied for transfer from one fusion partner to another. However, there are not any successful reports on the transfer of secondary metabolism related traits via somatic hybridization.

Carrot (*D. carota* var. *sativus* Hoffm., $2n = 18$), with the isoprenoid biosynthetic pathway, is an important member of family Apiaceae and is cultivated all over the world as a popular vegetable because of its high content of fat-soluble hydrocarbon and carotenoids. The carrot has also been a good experimental material for tissue culture because it was one of the first plants used for successful in vitro growth (Gautheret 1939). The utilization of liquid cultures for the cultivation of somatic cells in recapitulating embryogeny was reported as early as 1958 in carrots (Steward et al. 1958). From then on, it has become convenient to set up and maintain carrot cell lines. Moreover, the carrot could accept alien chromatins in somatic hybridization even they were derived from remote species (Kisaka et al. 1994). Therefore, the carrot genome might have the potential ability to provide the buffer for the improvement of secondary metabolism systems in somatic hybridization.

In this paper, the somatic hybridization between American ginseng and carrot were developed for the first time to study whether the ginsenosides could be produced by transferring genetic materials of American ginseng to the carrot genome.

Materials and methods

Induction and culture of calli and cell suspension

American ginseng seeds (donated by Shandong Laiyang Lvye American Ginseng Company, People's Republic of China) without capsules were sterilized in 75% ethanol for 3 min followed by 5 min in 0.1% $HgCl_2$, and then extensively washed with sterile water. The excised mature embryos were kept on MB solid medium, which was composed of MS macro- and micro-elements (Murashige and Skoog 1962), B5 vitamins (Gamborg et al. 1968), 2 mg/l glycine, 146 mg/l glutamine, 300 mg/l casein hydrolysate, 30 g/l sucrose and 7.5 g/l agar at pH 5.8, supplemented with 1 mg/l 2,4-Dichlorophenoxyacetic acid (2,4-D) and 0.1 mg/l kinetin, for about 4 weeks at 25°C in the dark for callus inducement. Yellow and granular calli

were obtained and then transferred onto the fresh MB solid medium supplied with 1 mg/l 2,4-D every 20 days. Carrot calli were induced from the cambium portion of the taproot from carrot (*D. carota* var. *sativus* Hoffm, purchased from retailers, Jinan, People's Republic of China). The sterilized cambium pieces (4 mm × 4 mm × 1 mm) were maintained on the MB_2 solid medium (Xia and Chen 1996) for 3 weeks in the dark at 25°C. Small and granular calli were selected and transferred to MB_2 liquid medium, shaking at the speed of 70 rpm for cell suspension. Suspension cells were sub-cultured every 7 days.

Protoplast isolation, fusion and culture

After 10 days of sub-culture on the solid medium, fresh calli of American ginseng were cut into tiny pieces and incubated in the enzyme solution (0.6 M mannitol, 5 mM $CaCl_2$, 1.5% cellulase onozyka RS and 0.3% pectolyase Y-23, sterilized through the 0.22 μm filter) for 4 h with gentle shaking at the speed of 70 rpm at room temperature. The protoplasts were filtered through a 50 μm sterilized falcial filter, collected by centrifugation at 100×*g* for 5 min, washed twice with 5 ml washing buffer (0.6 M mannitol and 5 mM $CaCl_2$, pH 5.8) and then adjusted with washing buffer to a density of 1×10^6 ml $^{-1}$ using the hemacytometer under compound microscope at 200× (10× × 20×). The same protocol was used for the preparation of carrot protoplasts which were derived from 4-day-subcultured cell suspension culture.

The ginseng and carrot protoplasts were mixed thoroughly at a ratio of 1:1. Each portion of 0.15–0.2 ml mixture was put on a 3.5–4.5 cm glass Petri dish to form a thin layer and stayed for 10–15 min. Cell fusion was performed using the polyethylene glycol (PEG) 6000 method as previously described (Xia and Chen 1996). After the cell fusion procedure, the petri dishes were sealed by Parafilm M (Pechiney Plastic Packaging Company, USA). The fusion protoplasts were cultured in the dark at 25°C in static P_5 liquid medium, which includes MS macro- and micro-elements, B5 vitamins, 2 mg/l glycine, 146 mg/l glutamine, 500 mg/l casein hydrolysate, 0.5 M glucose, 10 g/l sucrose and 1 mg/l 2,4-D at pH 5.8. The ginseng and carrot protoplasts were also cultured separately in static P_5 liquid medium as controls. The regenerated calli (about 2 mm in diameter after 7-week culture) were transferred onto the MB solid medium with 1 mg/l 2,4-D and sub-cultured every 2 weeks for proliferation. After 60 days of subculture, the calli were selected and transferred onto the MB solid medium with 1 mg/l 6-Benzylaminopurine (6-BA) and 1 mg/l indoleacetic acid (IAA) following exposure to 12 h of florescent light at 2,000 lx every day for differentiation.

Hybrid nature identification

Isozyme analysis

Fresh calli from putative hybrids and parents were ground in 0.1 M Tris–HCl (pH 8.3) at a ratio of 1:1 (e.g. 1 mg of plant material to 1 ml of buffer). The homogenates were centrifuged at 12,000 rpm for 10 min. Supernatants were electrophoresed with polyacrylamide gel electrophoresis (PAGE) in 3% spacer gel–10% separating gel. For peroxidase isozyme analysis, the gel was stained with 100 ml staining buffer containing 20 ml 2% Benzedrine, 70.4 mg vitamin C, 20 ml 0.6% H_2O_2 and 60 ml distilled water. For esterase analysis, the gel was stained with 90 ml phosphate buffer (pH 6.4) containing 90 mg fast blue B salt, 6 ml 2% alpha-naphthyl acetate and 3 ml 2% beta-naphthyl acetate (both dissolved in 1 ml acetone and diluted to 100 ml with 80% alcohol) (Hu and Wan 1985).

PCR analysis

DNA of the calli of putative hybrids and parent were extracted according to Doyle and Doyle (1990). Each 20 μl PCR solution contained 2 μl 10× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of dNTP, 50 ng template DNA, and 1 U *Taq* (Takara, People's Republic of China). For RAPD analysis, 0.4 μM RAPD primer (OPERON, USA) was included; for 5S rDNA spacer sequence analysis, 0.4 μM of each primer (p^{5s1}: 5′-GAGAGTAGTACATCGATGGG-3′; p^{5s2}: 5′-GGAGTTCTGACGGGATCCGG-3′) was included in the PCR solution (Cheng et al. 2004). The PCR program for RAPD: a denaturation step of 94°C for 5 min; 45 cycles of 94°C for 10 s, 36°C for 30 s, and 72°C for 1 min; a final incubation of 72°C for 10 min. The PCR program for amplifying the 5S rDNA spacer: 94°C for 5 min; followed with 35 cycles of 94°C for 1 min, 57°C for 1 min 20 s, and 72°C for 2 min; then 72°C for 10 min as a final extension step. Amplification products were electrophoresed in 1.5% (RAPD) and 2.5% (5S rDNA) agarose gels. Gels were stained with 0.5 μg/ml ethidium bromide and analyzed with Syngene gel imaging system (Syngene, USA).

Chromosome counting

Fresh calli of hybrids and parents were pretreated in water at 4°C for 24 h and then fixed at room temperature with acetic alcohol (ethanol:acetic acid = 3:1). After washing with distilled water, the fixed samples were softened in an enzyme mixture solution (1.5% cellulase onozyka RS, 0.5% pectolyase Y-23, 0.6 M mannitol, 5 mM $CaCl_2$, pH 5.8) for 30 min and then washed three times with distilled water. The softened samples were kept in the water for

0.5–1 h, and then they were spread on a grease-free glass slide and squashed to spread the chromosomes. Chromosome spreads were stained by a standard aceto carmine method (Buck 1935).

Genomic in situ hybridization (GISH)

Total genomic DNA of American ginseng was extracted and then labeled using Nick Translation Kit (catalog no. 1745816, Roche) following the manufacture's instructions. Slides of hybrid calli and parents with well spread chromosomes were selected for GISH as described previously (Xiang et al. 2003). The hybridization mixture (20 µl per slide) contained 50 ng of labeled American ginseng DNA probe and 2 µg of blocking DNA from carrot. Fluorescent images were captured using a fluorescence microscope (Nikon Eclipse E600).

High performance liquid chromatography (HPLC) analysis

The calli of hybrids and parents which were at the middle of the subculture period were dried at room temperature and ground into fine powder. About 0.1 g of the powder was separately sonicated for 0.5 h with 50 ml methanol and repeated three times. After cooling, methanol was added to make up the lost weight. The mixture was centrifuged at 1,500×*g* for 15 min, and then the methanol solution was collected and roto-evaporated to dryness. The residue was dissolved in water, followed by extraction with 50 ml of water-saturated *n*-BuOH (butanol) twice. The *n*-BuOH layer was evaporated under vacuum in a rotary evaporator. Each extract was dissolved in 10 ml methanol, filtered through a 0.2-µm nylon filter, and the final solution was analyzed.

The HPLC analysis was performed using a Prosphere C_{18} (5 µm, 250 mm × 4.6 mm) at 40°C. The mobile phase consisted of 70% water and 30% acetonitrile. The constant flow rate was 1 ml/min and the absorbance was measured at a wavelength of 203 nm. The sample injection volume was 20 µl. Each set of samples was repeated three times. The Rb_1 standard was purchased from National Institute for the Control of Pharmaceutical and Biological Products (Jinan, People's Republic of China).

Results

Putative hybrid calli derived from fusion products

The color of American ginseng callus used for isolation of protoplasts was yellow (Fig. 1a) and the suspension of carrot showed the golden-yellow color (Fig. 1b). As a control, the protoplasts derived calli of carrot (Fig. 1c) failed to differentiate; while the American ginseng protoplasts could not divide continuously: they could only grow into the small cell clumps with no further growth under the same culture condition. As for the fusion products, the first cell divisions of the fusion products were recognized on the fifth day after fusion and single cell clusters were formed after 30 days of culture (Fig. 1d). This kind of single cell clusters was compact and granular. Twenty-eight cell lines were proliferated from the single

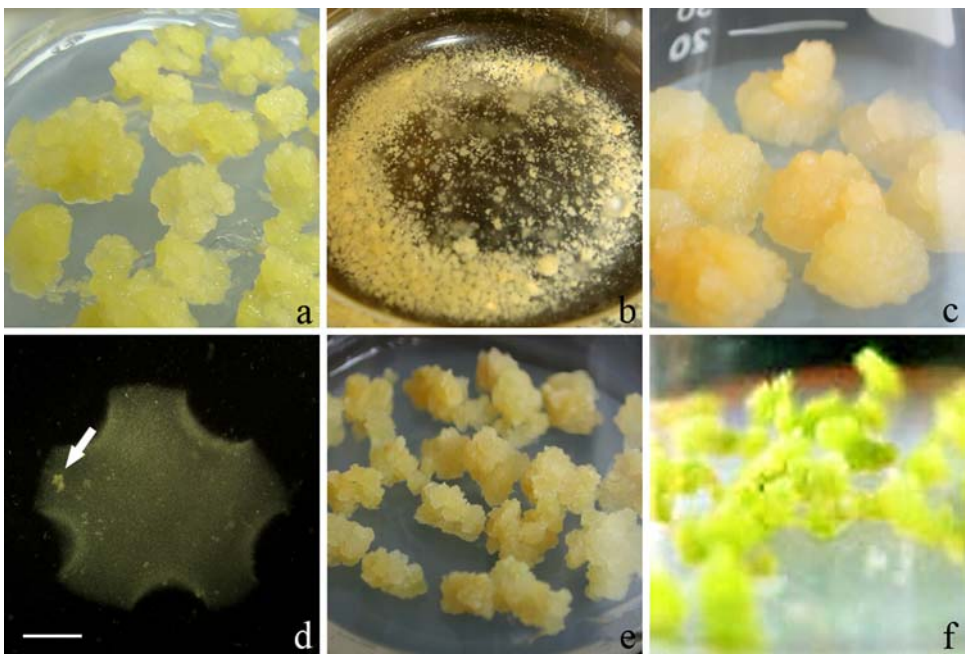

Fig. 1 Calli of parents and regenerated somatic hybrids. **a** American ginseng calli used for fusion. **b** Carrot suspension used for fusion. **c** Regenerated calli from carrot protoplast as a control. **d** The fused protoplasts in Petri dish after 30 days of culture. **e** Calli from fusion products without differentiation which was intermediate to colors of parents. **f** Regenerated fusion product calli with green spots. ➞: Regenerated single cell cluster; *Bar* 5 mm

cell clusters; they were light-yellow color (Fig. 1e) which was intermediate to that of parents or parent carrot-type color. After 2 months of subculture on differentiation medium, eight light-yellow of the 28 calli showed green spots (Fig. 1f) with no further regeneration of shoots or roots. The eight calli with the intermediate color and very primary differentiation ability from fusion products were considered as putative somatic hybrid calli and used for further characterization.

Hybrid nature of the putative hybrid calli

Isozyme pattern

To characterize the hybrid nature of the regenerated calli, peroxidase and esterase assays were conducted. All the eight calli, which were named no. 1–8, showed the similar pattern to that of parent carrot, but they still had a few American ginseng bands. They were subsequently identified as hybrids. As it is shown in Fig. 2a, calli no. 3–6 had the characteristic band of parent American ginseng. Calli no. 1 and 8 had American ginseng band, and calli no. 2 and 7 showed new bands on esterase analysis in Fig. 2b. These results suggested that both parents had taken part in the protoplast fusion, most of the carrot genetic materials remained in all the hybrid calli and American ginseng's genetic contents were included in some calli.

RAPD pattern

Out of 86 primers used, 14 can be used to distinguish the fusion parents (Table 1). All the eight hybrid calli identified by the isozyme analysis had the characteristic bands of carrot when amplified with different primers, which again demonstrated that the genetic constitutions of the hybrid calli were mostly from the carrot genome (Table 1). The introgression of American ginseng chromatin was revealed by the RAPD band(s) in calli no. 1, 2, 3, 5–8 amplified with six pairs of primers. Figure 3a demonstrates that all eight calli have the prominent bands from carrot and amplification result from callus no. 1, 3, 5, 6 and 8 show also prominent band(s) from American ginseng. Additionally, new bands were shown in calli no. 3–8. Figure 3b shows that no. 2 has American ginseng characterized band and no. 3–5, 7, and 8 show new band(s). Novel RAPD band(s) in all the eight calli suggested the parental DNA reset and sequence variation, genetic recombination or shuffling (Liu et al. 2007) between American ginseng and carrot (Fig. 3; Table 1).

5S rDNA profile

5S rDNA spacer sequences have been routinely used as a diagnostic marker for different plant species (Zanke et al. 1995). To further investigate the genetic constitution of the hybrid calli, 5S rDNA spacer sequences were amplified by

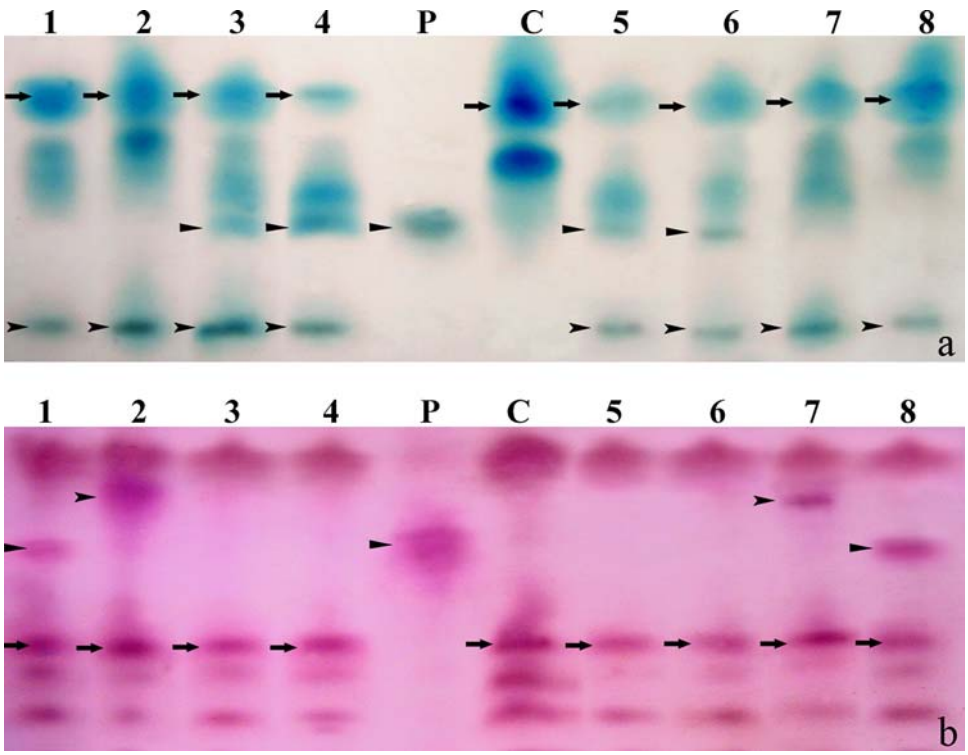

Fig. 2 Isozyme profiles of parents and hybrid calli. **a** Peroxidase profile. **b** Esterase profile. *P Panax quinquefolius* (American ginseng), *C Daucus carota* (carrot), *1–8* hybrid calli no. 1–8, ➞ parent carrot characteristic band, ▶ parent American ginseng characteristic band, ➤ novel band

Table 1 RAPD profiles of asymmetric somatic hybrid calli of carrot with American ginseng + carrot with various primers

Primers	Hybrid calli							
	1	2	3	4	5	6	7	8
OPA-01	PC	CN	PCN	CN	PCN	PCN	CN	PCN
OPA-08	C	C	C	C	C	C	C	C
OPA-17	PCN	PCN	CN	CN	PCN	PN	P	PCN
OPA-19	C	PCN	CN	CN	CN	C	CN	CN
OPA-06	C	C	C	C	C	C	C	C
OPF-05	N	N	PN	CN	PN	C	PC	PC
OPH-04	C	C	C	C	C	C	C	C
OPH-20	C	C	C	C	C	C	C	C
OPJ-19	C	C	C	C	C	C	C	C
OPM-01	PC	PC	C	CN	C	C	C	–
OPM-05	–	C	C	C	C	C	C	C
OPN-16	CN	C	CN	CN	–	C	CN	C
OPP-08	C	C	C	C	C	C	C	C
OPP-09	P	–	–	C	C	–	PC	–

P Containing band(s) of American ginseng, *C* containing band(s) of carrot, *N* containing novel band(s)

PCR in no. 1–8. PCR results showed that all the calli contained specific fragment(s) of carrot and no. 2 contained spacer sequence from American ginseng as well (Fig. 4). This showed DNA of American ginseng was infrequently transferred into specific genomic loci. The isozyme assay and PCR amplification with random and specific primers at least demonstrated that calli no. 1–8 are somatic hybrids though the genetic backgrounds of them are mainly from carrot. It is thus necessary to check the cytogenetic characters of these somatic hybrids.

Chromosome number and GISH karyotype

Chromosome numbers of carrot and American ginseng are 18 and 48, respectively (Punja et al. 2004; Hisaka et al. 1997). However, the chromosome number may vary due to long term culture, therefore, chromosome numbers of the parents and hybrid calli subcultured for 2–12 months were all inspected. The chromosome numbers were 40–48 in 70% of callus cells of American ginseng (Fig. 5a), and 18–20 in 80% of carrot suspension cells whose chromosome number ranged from 12 to 30 (Fig. 5b). As for the hybrid calli at different ages, the number of chromosomes mainly ranged from 18 to 28 (70% out of the cells analyzed), with the variation from 18 to 59 (Fig. 5c–f).

We further analyzed the genome constitution of the hybrid calli with GISH. GISH karyotypes of callus no. 1 and 8 were shown in Fig. 6. It was shown that no intact chromosomes but chromatin from American ginseng had been inserted randomly into carrot chromosomes (Fig. 6c, d). Similar results were observed in other hybrid calli. This demonstrated that limited genetic materials of American ginseng had been introgressed into the carrot genetic background.

Analysis of Rb_1 content in somatic hybrid calli

The content of the major ginsenoside Rb_1 was quantified by HPLC as shown in Figs. 7 and 8. Compared with the parent American ginseng calli with content of 1.60 ± 0.05 mg/g (mean ± standard error from three independent experiments), Rb_1 content was found to exist in hybrid calli no. 1–8 with different concentration (1.74 ± 0.19, 1.24 ± 0.07,

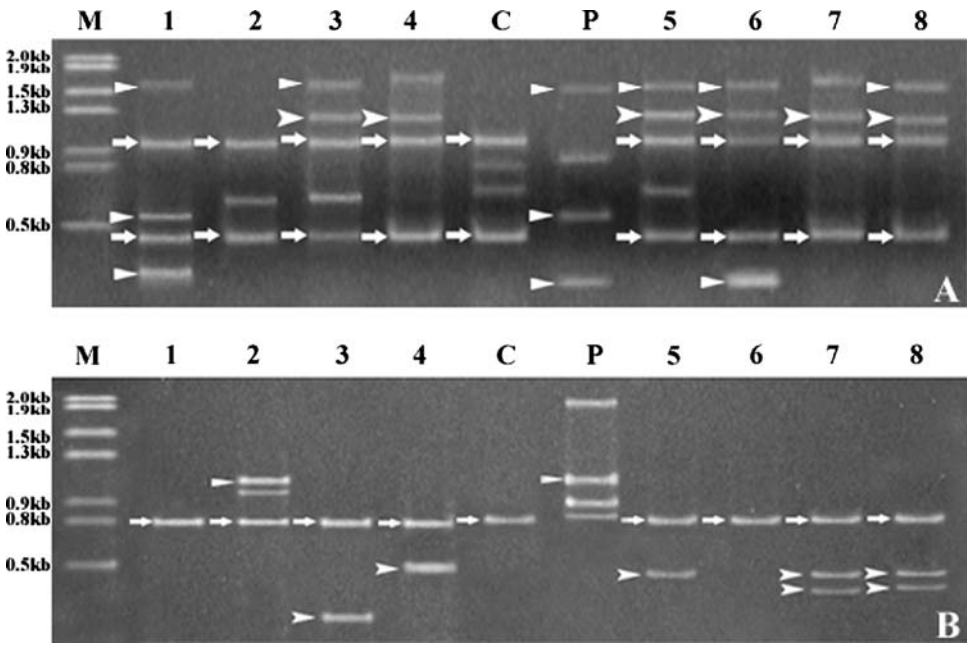

Fig. 3 RAPD profile of parents and hybrid calli. **a** Profile amplified with primer OPA-01. **b** Profile amplified with primer OPA-19. *M* λDNA digested with *Hind*III and *Eco*RI, *P* calli of American ginseng, *C* calli of carrot, *1–8* calli of hybrids, ➙ fragment characteristic of parent carrot, ▶ fragment characteristic of parent American ginseng, ➤ novel fragment

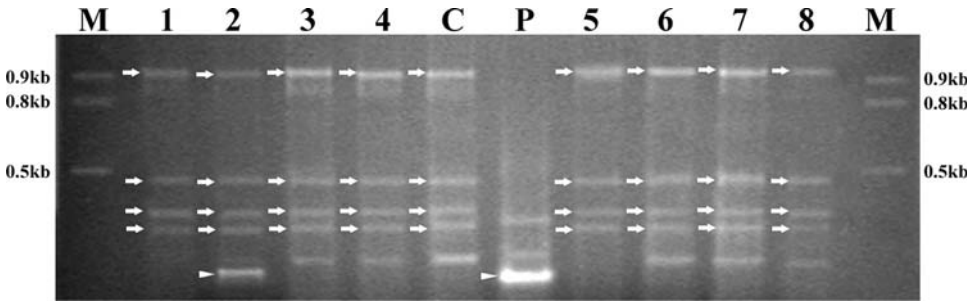

Fig. 4 Profile of 5S rDNA spacer sequences. *M* λDNA digested with *Hin*dIII and *Eco*RI, *P* calli of American ginseng, *C* calli of carrot, *1–8* hybrid calli, ➙ fragment characteristic of parent carrot, ▶ fragment characteristic of parent American ginseng

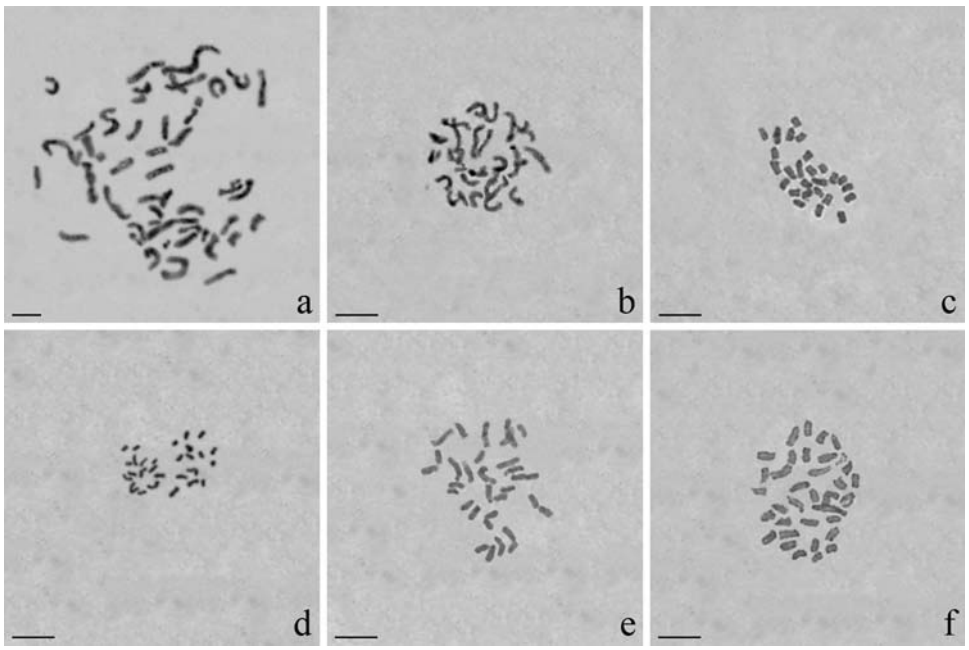

Fig. 5 Chromosomes of parents and hybrid calli. **a** American ginseng, $2n = 48$. **b** Carrot, $2n = 18$. **c** Chromosome number of calli no. 1, $2n = 26$. **d** Chromosome number of calli no. 2, $2n = 28$. **e** Chromosome number of calli no. 4, $2n = 28$. **f** Chromosome number of calli no. 8, $2n = 34$. *Bar* 5 μm

0.97 ± 0.06, 1.00 ± 0.13, 0.59 ± 0.14, 0.96 ± 0.09, 1.68 ± 0.09 mg/g), except in no. 5 and the parent carrot.

Discussion

Based on the results of cytology, biochemical and molecular markers, we confirmed that elimination and introgression of the American ginseng genetic material had occurred during the somatic hybridization with carrot, and highly asymmetric hybrid calli were obtained. It has been reported that the chromosome exclusion and elimination often happens between genomes of parents with distant phylogenetic relationships, and that parental genome in the interphase could also be usually excluded by the other parental genomes during mitosis (Nakano et al. 1996; Navrátilová et al. 1997; Hu et al. 2002; Dudits 1988). The chromosome exclusion and elimination lessened parental genome incompatibility and hence asymmetric hybrids were generated. In our experiments, both the phylogenetic relationship between American ginseng and carrot and the asynchronous cell cycle behavior were likely responsible for the high asymmetry of the hybrid calli. Concerning the cell cycle behavior, American ginseng protoplasts, which could not divide continuously as a control, were inferred as in the interphase when they were fused with carrot protoplasts at the active stage of mitosis.

As it has been previously reported, some highly asymmetric somatic hybrid calli could be regenerated in remote fusion combinations via complementation of parental genomes (Xia and Chen 1996; Wang et al. 2005; Zhou et al. 2006; Deng et al. 2007). In the present study, the control cells from the bi-parent protoplasts could not divide or differentiate. Therefore, hybrid calli with the primary differentiating ability of generating green spots should come from the genetic complementation of both parents. The phenomenon that the hybrid calli could not differentiate into complete plants via the complementation may be ascribed to some related genes which have been excluded or lost in the duration of tissue culture or were not introgressed from American ginseng to the hybrids.

The correlation between the culture time and the accumulation of chromosome variations was first documented in *D. carota* (Smith and Street 1974). Since then, some

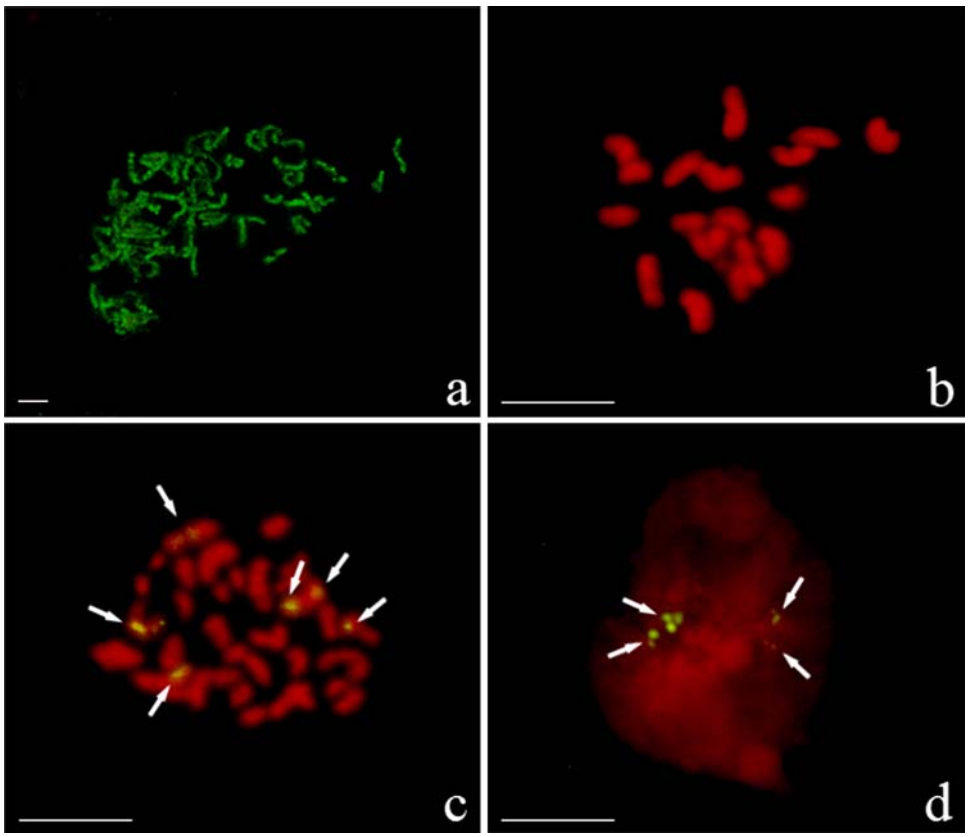

Fig. 6 Analysis of hybrid calli by GISH. Total genome DNA of American ginseng was labeled as a probe using digoxigenin and detected with FITC. The chromosomes were counterstained with propidium iodide. **a** American ginseng. **b** Carrot. **c** Hybrid callus no. 1 showing six chromosome fragments of American ginseng added to the genome of carrot. **d** Nucleus of hybrid callus no. 8 showing insertion of American ginseng chromosome fragments. →: Chromosome fragment of American ginseng. *Bar* 5 μm

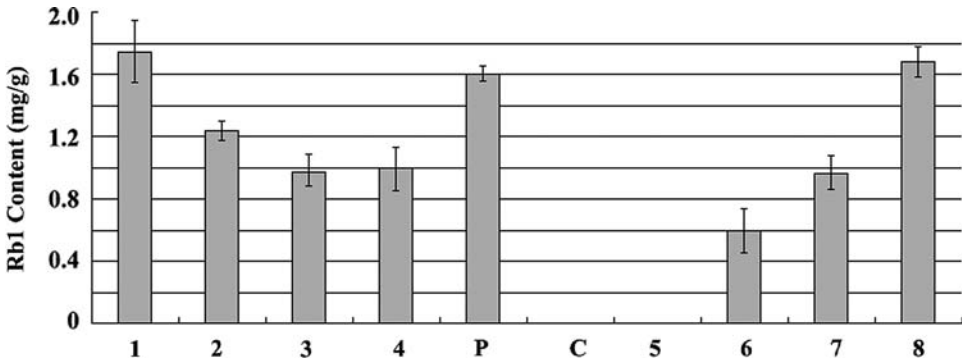

Fig. 7 Analysis of Rb_1 content in calli of hybrids and parents. *P* Calli of American ginseng, *C* calli of carrot, *1–8* calli of hybrid. *Vertical bars* represent standard error of mean of three replicate experiments

studies about chromosomal variations were observed in cultured calli of *Nicotiana tabacum*, potato cultivars, *Lotus corniculatus*, rice and other species. There is also evidence that polyploidy occurs during the long term course of culture (Niizeki and Lu 2003; Song et al. 2000). In this case, the chromosome numbers of the hybrid calli and carrot suspension cells, which were selected from different culture period from 2 to 12 months were examined. We found that 70% of hybrid cells showed chromosome number ranging from 18 to 28 and 80% of carrot suspension cells contained 18–20 chromosomes. This result suggests that the chromosome numbers of hybrid calli were statistically steady even though some chromosome variations were inevitable to some extent. The GISH karyotypes of cells from hybrid calli further showed hybridizing signals of American ginseng at the chromosome of carrot (Fig. 6). The result reveals that: (1) the increased chromosome number of hybrid calli was not caused by the insertion of the contact chromosome of American ginseng but probably because of polyploidy during the process of tissue culture; (2) only the small inserted chromosome fragments of American ginseng were detected, which indicates that the inserted foreign chromosome fragments have the predominance to be preserved in the heterogeneous genome by the way of taking part in normal cell divisions.

The commercial value of American ginseng is the contents of many biologically active compounds, especially

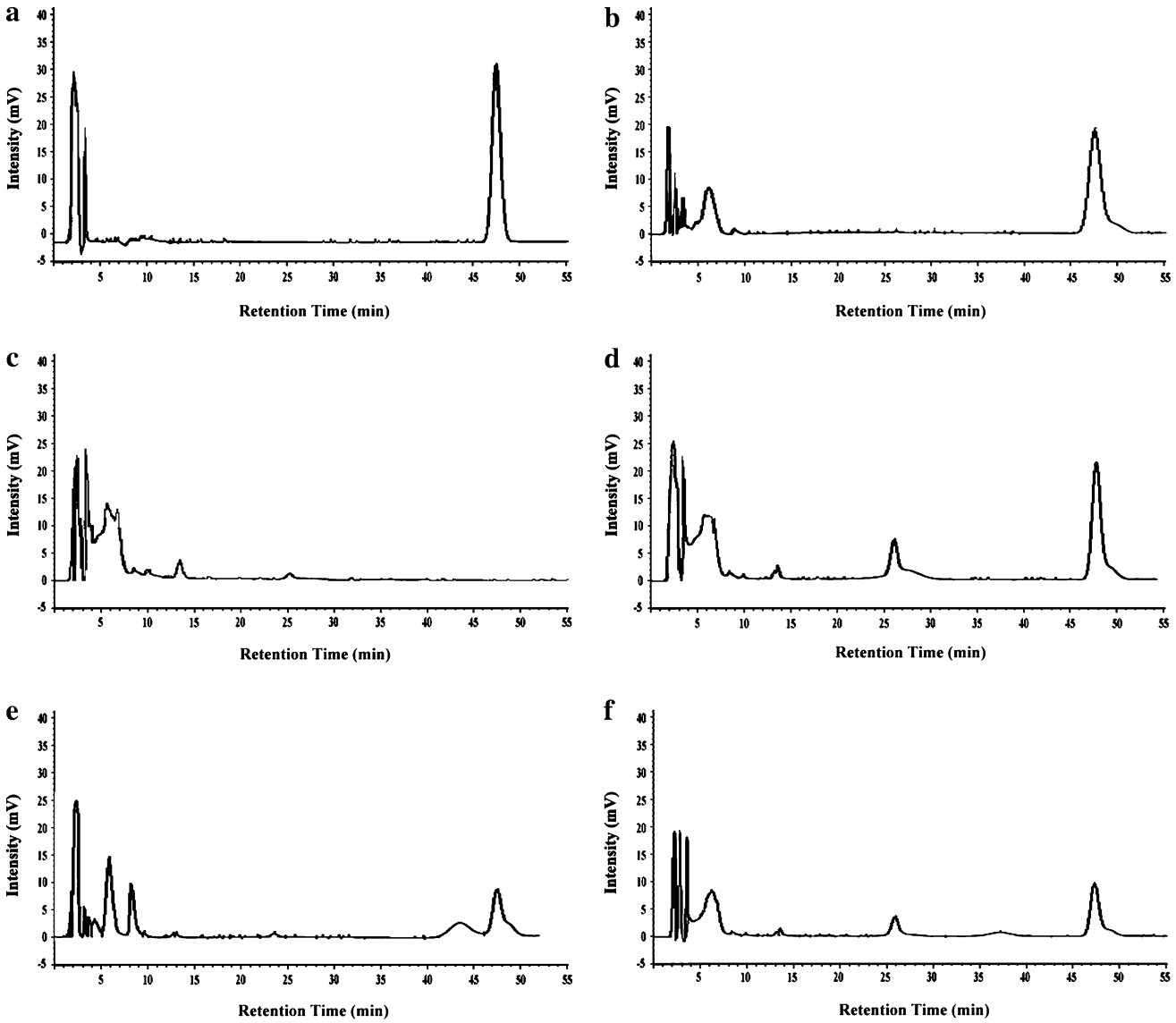

Fig. 8 HPLC chromatogram of calli of hybrids and parents. **a** Rb_1 standard. **b** Calli of American ginseng. **c** Calli of carrot. **d** Calli of hybrid no. 1. **e** Calli of hybrid no. 6. **f** Calli of hybrid no. 7

ginsenosides. Ginsenosides are a big group, including major and minor ginsenosides. But the ginsenoside concentrations and the percentages are dependent on the genotypes and variable in different parts of American ginseng and could also be easily affected by the cultivation conditions (Yuan et al. 2002). Among these ginsenoside monomers, Rb_1 occupies a relatively steady and high ratio and plays an important role in pharmacological effects. Animal experiments showed that Rb_1 has multiple functions such as anti-inflammatory (Smolinski and Pestka 2003), partial neurotrophic and neuroprotective actions (Himi et al. 1989), and potentially effective therapeutic agents for spinal cord injuries (Liao et al. 2002). Because of the high level of content and important pharmacological value, it is desirable for us to check the existence of Rb_1 in somatic hybrid calli for primary investigation.

With a view to ginsenoside Rb_1 synthesis, oxidosqualene cyclase gene producing dammarenediol II synthase, genes related with dammarenediol synthase and many glucosyltransferase genes may be expressed at the same time (Jung et al. 2003; Tansakul et al. 2006). However, little is known about the biochemical synthesis pathway of ginsenoside in American ginseng. The genetic material of American ginseng introgressed into all hybrid calli probably includes these related genes. Although carrot has different kinds of secondary metabolic products and do not produce ginsenoside Rb_1, it also has the isoprenoid biosynthetic pathway. After alien genes related to a different

isoprenoid biosynthesis were transferred into carrot genome, the original isoprenoid biosynthetic pathway in carrot might be disturbed and switched, thus resulting in the accumulation of Rb_1. The hybrid calli contained different outputs of ginsenoside Rb_1 and even other unidentified secondary metabolic productions as shown in the HPLC chromatogram. The mechanism of accumulations of these productions is not clear yet, but this result gives us a new point of view of plant metabolic pathways—they have the potential possibility of communicating with each other.

Recently, cell suspensions and transformed roots (hairy roots) were reported as culture materials for the research on ginseng bioreactor establishment. Ginseng cells grow very slowly and need a lot of optimization of culture mediums (Mathur et al. 1994; Thanh et al. 2005). Commercial-scale bioreactor culture of ginseng was rarely reported although there are lots of research efforts on ginsenoside production using cell bioreactor systems (Asaka et al. 1993; Choi et al. 2003). Consequently, transformed root (hairy root) culture of American ginseng is an alternative method that can avoid the above problems (Yoshikawa and Furuya 1987). But it is known that the biological safety of transgenic plants, a large group of genetically modified organisms (GMO), has been argued worldwide for a long time because of the unclear potentials for human allergenicity and increase in the antibiotic resistance of human pathogens (Gay and Gillespie 2005; Hodgson 2001). The attitude about the products of transgenic plants with antibiotic resistance genes is depended on the consumer acceptability to some extent. Therefore, crude extracts of hairy roots by bioreactor culture still need to be proven safe to mammalian cells before this approach is accepted by consumers for commercial mass. Somatic hybridization is an alternative approach to obtain the novel germplasm. The parents applied for somatic hybridization can be derived from natural species; therefore, at least, the contamination of antibiotic resistance markers can be avoided in fusion products, although somatic hybrids are also considered as GMO in some countries.

Compared with American ginseng, history of research on the tissue culture of carrot is much longer and better developed (Steward et al. 1958; Halperin and Wetherell 1964). We initially planned to take advantages of carrot tissue culture to regenerate carrot root with ginsenosides. Even though no hybrid plants were obtained, this primary investigation strongly indicates the possibility to produce the ginsenoside Rb_1 heterogeneously. We may make use of the rapid growth of carrot cell lines to produce ginsenosides more efficiently.

In conclusion, we obtained asymmetric somatic hybrid calli of carrot with American ginseng which could accumulate ginsenoside Rb_1. Further, we will try to improve the differentiation ability of obtained somatic hybrid calli to get the root, shoot and/or hybrid plants for more investigation and exploitation of their production of ginsenosides. There were strong genetic differences between wild and cultivated populations even within populations (Grubbs and Case 2004). These variations were also found in the productions of different ginsenoside among populations of wild and cultivated American ginsengs which could be influenced by genotype and/or location (Li et al. 1996; Lim et al. 2005; Schlag and McIntosh 2006). Therefore, we should select the genotype of American ginseng whose calli could produce a high content of ginsenosides as the parent for further somatic hybridization. Moreover, in this preliminary experiment, we focused on the major ginsenoside Rb_1 content of the somatic hybrids. Other important ginsenosides, such as Rb_2, Rc, Rd, Re, Rg_1, need to be examined after new somatic hybrid calli are obtained. It is also important to detect the related genes so as to explain the molecular mechanism of the ginsenoside production in the somatic hybrids.

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References

Ali MB, Hahn EJ, Paek KY (2006) Copper-induced changes in the growth, oxidative metabolism, and saponin production in suspension culture roots of *Panax ginseng* in bioreactors. Plant Cell Rep 25:1122–1132

Asaka I, Ii I, Hirotani M, Asada Y, Furuya T (1993) Production of ginsenoside saponins by culturing ginseng (*Panax ginseng*) embryogenic tissues in bioreactors. Biotechnol Lett 15:1259–1264

Brewer EP, Saunders JA, Angle JS, Chaney RL, McIntosh MS (1999) Somatic hybridization between the zinc accumulator *Thlaspi caerulescens* and *Brassica napus*. Theor Appl Genet 99:761–771

Buck JB (1935) Permanent aceto-carmine preparations. Science 81:75

Cheng A, Xia G, Zhi D, Chen H (2004) Intermediate fertile *Triticum aestivum* (+) *Agropyron elongatum* somatic hybrids are generated by low doses of UV irradiation. Cell Res 14:86–91

Choi YE, Jeong JH, Shin CK (2003) Hormone-independent embryogenic callus production from ginseng cotyledons using high concentrations of NH_4NO_3 and progress towards bioreactor production. Plant Cell Tiss Organ Cult 72:229–235

Chuong PV, Beversdorf WD, Powell AD, Pauls KP (1988) The use of haploid protoplast fusion to combine cytoplasmic atrazine resistance and cytoplasmic male sterility in *Brassica napus*. Plant Cell Tiss Organ Cult 12:181–184

Court WA, Reynolds LB, Hendel JG (1996) Influence of root age on the concentration of ginsenosides of American ginseng (*Panax quintuefolium*). Can J Plant Sci 76:853–855

Davey MR, Anthony P, Power JB, Lowe KC (2005) Plant protoplasts: status and biotechnological perspectives. Biotechnol Adv 23:131–171

Deng J, Cui H, Zhi D, Zhou C, Xia G (2007) Analysis of remote asymmetric somatic hybrids between common wheat and *Arabidopsis thaliana*. Plant Cell Rep 26:1233–1241

Doyle JJ, Doyle DJ (1990) Isolation of plant DNA from fresh tissue. Focus 12:13–15

Dudits D (1988) Increase of genetic variability by asymmetric cell hybridization and isolated chromosome transfer. Plant Cell Tiss Organ Cult 12:205–211

Dudits D, Maroy E, Praznovszky T, Olah Z, Gyorgyey J, Cella R (1987) Transfer of resistance traits from carrot into tobacco by asymmetric somatic hybridization: regeneration of fertile plants. Proc Natl Acad Sci USA 84:8434–8438

Fournier AR, Proctor JT, Gauthier L, Khanizadeh S, Belanger A, Gosselin A, Dorais M (2003) Understory light and root ginsenosides in forest-grown *Panax quinquefolius*. Phytochemistry 63:777–782

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50:151–158

Gautheret RJ (1939) Sur la possibilité de réaliser la culture indefinie des tissues de tubercules de carotte. C R Acad Sci 208:218–220

Gay PB, Gillespie SH (2005) Antibiotic resistance markers in genetically modified plants: a risk to human health? Lancet Infect Dis 5:637–646

Grubbs HJ, Case MA (2004) Allozyme variation in American ginseng (*Panax quinquefolius* L.): variation, breeding system, and implications for current conservation practice. Conserv Genet 11:13–23

Halperin W, Wetherell DF (1964) Adventive embryony in tissue cultures of the wild carrot, *Daucus Carota*. Am J Bot 51:274–283

Himi T, Saito H, Nishiyama N (1989) Effect of ginseng saponins on the survival of cerebral cortex neurons in cell cultures. Chem Pharm Bull (Tokyo) 37:481–484

Hisaka H, Kisaka M, Kanno A, Kameya T (1997) Production and analysis of plants that are somatic hybrids of barley (*Hordeum vulgare* L.) and carrot (*Daucus carota* L.). Theor Appl Genet 94:221–226

Hodgson E (2001) Genetically modified plants and human health risks: can additional research reduce uncertainties and increase public confidence. Toxicol Sci 63:153–156

Hu N, Wan X (1985) Application of isozyme technique. Hunan Science and Technology Press, Changsha, pp 74–76 (in Chinese)

Hu Q, Andersen SB, Dixelius C, Hansen LN (2002) Production of fertile intergeneric somatic hybrids between *Brassica napus* and *Sinapis arvensis* for the enrichment of the rapeseed gene pool. Plant Cell Rep 21:147–152

Jackson CJC, Dini JP, Lavandier C, Faulkner H, Rupasinghe HPV, Proctor JTA (2003) Ginsenoside content of North American ginseng (*Panax quinquedolius* L. Araliaceae) in relation to plant development and growing locations. J Genseng Res 27:135–140

Jian X, Ping L, Tezuka Y, Namba T, Kadota S, Zhong JJ, Wang S (1998) Effects of nitrogen source on the production of ginseng saponin and polysaccharide by cell cultures of *Panax quinquefolium*. Process Biochem 33:671–675

Jung JD, Park HW, Hahn Y, Hur CG, In DS, Chung HJ, Liu JR, Choi DW (2003) Discovery of genes for ginsenoside biosynthesis by analysis of ginseng expressed sequence tags. Plant Cell Rep 22:224–230

Kim YS, Hahn EJ, Murthy HN, Paek KY (2004) Adventitious root growth and ginsenoside accumulation in *Panax ginseng* cultures as affected by methyl jasmonate. Biotechnol Lett 26:1619–1622

Kisaka H, Lee H, Kisaka M, Kanno A, Kang K, Kameya T (1994) Production and analysis of asymmetric hybrid plants between monocotyledon (*Oryza sativa* L.) and dicotyledon (*Daucus carota* L.). Theor Appl Genet 89:365–371

Li TSC, Mazza G, Cottrell AC, Gao L (1996) Ginsenosides in roots and leaves of American ginseng. J Agr Food Chem 44:717–720

Liao B, Newmark H, Zhou R (2002) Neuroprotective effects of ginseng total saponins and ginsenosides Rb_1 and Rg_1 on spinal cord neurons in vitro. Exp Neurol 173:224–234

Lim W, Mudge KW, Vermeylen F (2005) Effects of population, age, and cultivation methods on ginsenoside content of wild American ginseng (*Panax quinquefolium*). J Agric Food Chem 53:8498–8505

Liu S, Zhao S, Chen F, Xia G (2007) Generation of novel high quality HMW-GS genes in two introgression lines of *Triticum aestivum/Agropyron elongatum*. BMC Evol Biol 7:76–83

Mathur A, Shukla YN, Pal M, Ahuja PS, Uniyal GC (1994) Saponin production in callus and cell suspension cultures of *panax quinquefolium*. Phytochemistry 35:1221–1225

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Nakano M, Hoshino Y, Mii M (1996) Intergeneric somatic hybrid plantlets between *Dianthus barbatus* and *Gypsophila paniculata* obtained by electrofusion. Theor Appl Genet 92:170–172

Navrátilová B, Bůžek J, Široký J, Havránek P (1997) Construction of intergeneric somatic hybrids between *Brassica oleracea* and *Armoracia rusticana*. Biologia Plantarum 39:531–541

Niizeki M, Lu Z (2003) Somaclonal variation as a tool for plant breeding and genetics. Bull Fac Agric Life Sci Hirosaki Univ 6:1–17

Proctor JTA, Bailey WG (1987) Ginseng: industry, botany, and culture. Hort Rev 9:187–236

Punja Z, Feeney M, Schluter C, Tautorus T (2004) Multiplication and germination of somatic embryos of American ginseng derived from suspension cultures and biochemical and molecular analyses of plantlets. In Vitro Cell Dev Biol Plant 40:329–338

Schlag EM, McIntosh MS (2006) Ginsenoside content and variation among and within American ginseng (*Panax quinquefolius* L.) populations. Phytochemistry 67:1510–1519

Smith SM, Street HE (1974) The decline of embryogenic potential as callus and suspension cultures of carrot (*Daucus carota* L.) are serially subcultured. Ann Bot 38:233–241

Smolinski AT, Pestka JJ (2003) Modulation of lipopolysaccharide-induced proinflammatory cytokine production in vitro and in vivo by the herbal constituents apigenin (chamomile), ginsenoside Rb_1 (ginseng) and parthenolide (feverfew). Food Chem Toxicol 41:1381–1890

Song X, Xia G, Chen H (2000) Chromosomal variation in long-term cultures of several related plants in Triticinae. Acta Phytophysiol Sinica 26:33–38

Steward FC, Mapes MO, Mears K (1958) Growth and organized development of cultured cells. II. Organization in cultures grown from freely suspended cells. Am J Bot 45:705–708

Tansakul P, Shibuya M, Kushiro T, Ebizuka Y (2006) Dammarenediol-II synthase, the first dedicated enzyme for ginsenoside biosynthesis, in *Panax ginseng*. FEBS Lett 580:5143–5149

Teng WL, Sin T, Teng MC (2002) Explant preparation affects culture initiation and regeneration of *Panax ginseng* and *Panax quinquedolius*. Plant Cell Tiss Organ Cult 68:233–239

Thanh NT, Murthy HN, Yu KW, Hahn EJ, Paek KY (2005) Methyl jasmonate elicitation enhanced synthesis of ginsenoside by cell suspension cultures of *Panax ginseng* in 5-l balloon type bubble bioreactors. Appl Microbiol Biotechnol 67:197–201

Vazquez-Thello A, Yang JL, Hidaka M, Uozumi T (1996) Inherited chilling tolerance in somatic hybrids of transgenic *Hibiscus rosa-sinensis* × transgenic *Lavatera thuringiaca* selected by double-antibiotic resistance. Plant Cell Rep 15:506–511

Wang M, Xia G, Peng Z (2005) High UV-tolerance with introgression hybrid formation of *Bupleurum scorzonerifolium* Willd. Plant Sci 168:593–600

Wu J, Zhong JJ (1999) Production of ginseng and its bioactive components in plant cell culture: current technological and applied aspects. J Biotechnol 68:89–99

Xia G, Chen H (1996) Plant regeneration from intergeneric somatic hybridization between *Triticum aestivum* L. and *Leymus chinensis* (Trin) Tzvel. Plant Sci 120:197–203

Xiang F, Xia G, Chen H (2003) Effect of UV dosage on somatic hybridization between common wheat (*Triticum aestivumum* L.) and *Avena sativa* L. Plant Sci 164:697–707

Yoshikawa T, Furuya T (1987) Saponin production by cultures of *Panax ginseng* transformed with *Agrobacterium rhizogenes*. Plant Cell Rep 6:449–453

Yu KW, Hahn EJ, Paek KY (2000a) Production of adventitious ginseng roots using bioreactors. Korean J Plant Tissue Cult 27:309–315

Yu KW, Gao WY, Son SH, Paek KY (2000b) Improvement of ginsenoside production by jasmonic acid and some other elicitors in hairy root culture of ginseng (*Panax ginseng* C.A. Meyer). In Vitro Cell Dev Biol Plant 36:424–428

Yuan CS, Wu JA, Osinski J (2002) Ginsenoside variability in American ginseng samples. Am J Clin Nutr 75:600–601

Zanke C, Borisjuk N, Ruoss B, Schilde-Rentschler L, Ninnemann H, Hemleden V (1995) A specific oligonucleotide of the 5S-rDNA spacer and species-specific elements identify symmetric somatic hybrids between *Solanum tuberosum* and *S.* pinnatisectum. Theor Appl Genet 90:720–726

Zhou S, Brown DC (2006) High efficiency plant production of North American ginseng via somatic embryogenesis from cotyledon explants. Plant Cell Rep 25:166–173

Zhou C, Xia G, Zhi D, Chen Y (2006) Genetic characterization of asymmetric somatic hybrids between *Bupleurum scorzonerifolium* Willd and *Triticum aestivum* L.: potential application to the study of the wheat genome. Planta 223:714–724