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High-efficiency plant production via direct somatic single embryogenesis from preplasmolysed cotyledons of *Panax ginseng* and possible dormancy of somatic embryos

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Abstract Cotyledon explants of immature ginseng zygotic embryos cultured on Murashige and Skoog medium lacking growth regulators formed somatic embryos directly, most in a multiple state, fused together and to the parent cotyledon explants. When the cotyledon explants of ginseng were pretreated with 1.0 M sucrose for 24–72 h, all the somatic embryos developed individually from all surfaces of the cotyledons and the number of somatic embryos per explant was enhanced fourfold. Histological observation revealed that all the single somatic embryos from preplasmolysed cotyledons originated from epidermal single cells, whereas all the multiple embryos from cotyledons without pretreatment originated from epidermal and subepidermal cell masses. When the somatic embryos matured to the cotyledonary stage, further growth ceased and they remained white, probably indicating dormancy. Gibberellic acid (GA_3) (over 1.0 mg/l) or chilling treatment (–2°C for over 8 weeks) were prerequisites for the germination of somatic embryos. Ultrastructural observation revealed that the cotyledon cells of somatic embryos without chilling or GA_3 treatment contained numerous lipid reserves, dense cytoplasm, proplastids and non-activated mitochondria, whereas the cotyledon cells of somatic embryos after chilling or GA_3 treatment were highly vacuolated and contained well-developed chloroplasts and active-state mitochondria enclosing numerous cristae, indicating that in-vitro-developed somatic embryos of *P. ginseng* may be dormant after maturing in a manner similar to zygotic embryos.

Key words Dormant somatic embryo · Chilling treatment · Germination · Plasmolysis · *Panax ginseng*

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Abbreviations *BAP* 6-Benzylaminopurine · GA_3 Gibberellic acid · *MS medium* Murashige and Skoog medium

Introduction

Ginseng is a perennial herbaceous plant which grows very slowly: its cultivation requires of more than 3 years to produce seeds. Therefore, tissue culture procedures could contribute to clonal propagation and genetic breeding of this plant. Several papers have reported plant regeneration of ginseng through somatic embryogenesis (Butenko et al. 1968; Chang and Hsing 1980; Arya et al. 1991; Choi et al. 1998). It has been accepted that regeneration of plants with a well-developed root system from somatic embryos of ginseng is still very recalcitrant. Many investigations of ginseng tissue culture have recorded abnormal morphology of somatic embryos and multicotyledonary or fused somatic embryos (Butenko et al. 1968; Chang and Hsing 1980; Cellarova et al. 1992; Arya et al. 1993; Choi et al. 1998). It has been reported that the morphologically abnormal multiple and fused somatic embryos formed from ginseng cotyledon cultures only regenerate multiple shoots, whereas single somatic embryos regenerate into plants with well-developed roots and shoots (Choi et al. 1998). Generally, in direct somatic embryogenesis, somatic multiple embryos originate from multiple cell masses of cultured explants, while single embryos are derived from single cells (Williams and Maheswaran 1986; Choi and Soh 1994). Single or multiple embryo formation seems to depend on whether the embryogenic cells within the tissues are physiologically isolated or in a mass. Choi and Soh (1997) reported that plasmolysis pretreatment of cotyledon tissues of *Panax ginseng* can enhance single-embryo formation by disrupting plasmodesmata. The present experiment was carried out to establish high-efficiency plant production from ginseng cotyledon cultures using plasmolysis pretreatment.

Zygotic seeds of *P. ginseng* require a chilling treatment of about 3 months to break dormancy (Kuribayashi and

Ohashi 1971). In somatic embryos of *P. ginseng*, the combination of cytokinin 6-benzylaminopurine (BAP) and gibberellic acid (GA_3) are highly efficient for plant development from somatic embryos (Chang and Hsing 1980; Shoyama et al. 1987; Arya et al. 1993; Choi et al. 1998). However, it is not known whether somatic embryos of *P. ginseng* have dormancy characters similar to the zygotic embryos in seeds.

The present paper describes high-frequency plant production via direct somatic single-embryo formation from ginseng cotyledons by plasmolyzing pretreatment and the possible dormancy of somatic embryos in *P. ginseng*.

Materials and methods

Korean ginseng (*P. ginseng* C. A. Meyer) seeds contain extremely immature embryos just after harvest, and were stratified in humidified sand to mature. After seed coats had been dehusked, the seeds, in which zygotic embryos were in an immature state (3 mm in length), were immersed in 70% alcohol for 1 min, surface-sterilized in 1% NaOCl for 20 min, washed three times with sterilized distilled water, and the immature zygotic embryos were then dissected out.

For plasmolyzing pretreatment, cotyledon bases of zygotic embryos were transversally cut, and the excised cotyledons were pretreated in 1.0 M sucrose solution for 24–72 h, deplasmolyzed by additio of 0.1 M sucrose solution at 50% dilution for 5 min, and finally placed in a 0.1 M sucrose solution. After this treatment, the pretreated cotyledons were placed with their adaxial sides on the surface of the medium. The medium comprised hormone-free Murashige and Skoog (1962) (MS) medium containing 0.1 M sucrose and 0.7% agar. The medium was adjusted to pH 5.8 before autoclaving at 120°C for 15 min. Cotyledons were cultured in 10×2 cm petri dishes containing 30 ml of medium. Fifteen cotyledon explants were cultured on each petri dish. Triple replicates were used per treatment, and the experiments were repeated three times. The culture room was maintained at 24±2°C with a 16-h photoperiod of 24 μmol $m^{-2} s^{-1}$ under cool-white fluorescent tubes. The production rate of somatic embryos was evaluated by counting cotyledon explants producing somatic embryos after 10 weeks of culture. In addition, the number of somatic embryos per explant and the pattern and distribution of single and multiple embryos were also evaluated.

To investigate the effect of GA_3 on the germination of somatic embryos, cotyledonary somatic embryos were transferred to MS medium with various concentrations of GA_3 (0, 1, 4, 8, 10 mg/l), with 3% sucrose and 0.7% agar. After 2 months of culture, germination of somatic embryos was examined. Somatic embryos were cultured in 10×2 cm petri dishes. About 20 explants were used per treatment; this was repeated three times.

To investigate the effect of chilling treatment on the germination of somatic embryos, petri dishes containing somatic embryos were transferred to a low-temperature incubator for 2–12 weeks. Temperature regimes were adjusted to various levels (–2, 0, 4°C). After this treatment, the petri dishes were transferred to an incubation room. After 2 months of culture, the germination of somatic embryos was examined.

Germinating embryos after chilling or GA_3 treatment were manually removed from parent cotyledon explants by forceps, and cultured on 1/3-strength MS basal medium to support their continued growth into plantlets.

For histological observations of somatic embryo development from cotyledon explants with or without plasmolyzing pretreatment, cotyledon explants with somatic embryos at all stages of development were fixed in formalin:acetic acid:ethyl alcohol, dehydrated in ethyl alcohol and then embedded in paraplast. The samples were cut to 10 μm with a rotary microtome, and the sections stained with hematoxylin.

To observe ultrastructurally the cellular events after chilling (–2°C for 8 weeks) and GA_3 treatment (8 mg/l for 1 month), cotyledon segments of 1 mm^3 were sampled from the resting somatic embryos or from green somatic embryos after chilling or GA_3 treatment. Sampled cotyledon tissues were fixed in 1% glutaraldehyde (phosphate buffer pH 6.8) for 4 h at 4°C and postfixed in 2% OsO_4 for 2 h. The samples were dehydrated with ethyl alcohol and embedded in Epon resin. The ultrathin sections were cut from median longitudinal sections of cotyledons using an LKB-V ultramicrotome. Thin sections were stained with 1% uranyl acetate and lead citrate. The sections were observed by transmission electron microscope (JEM 1200 EX-II).

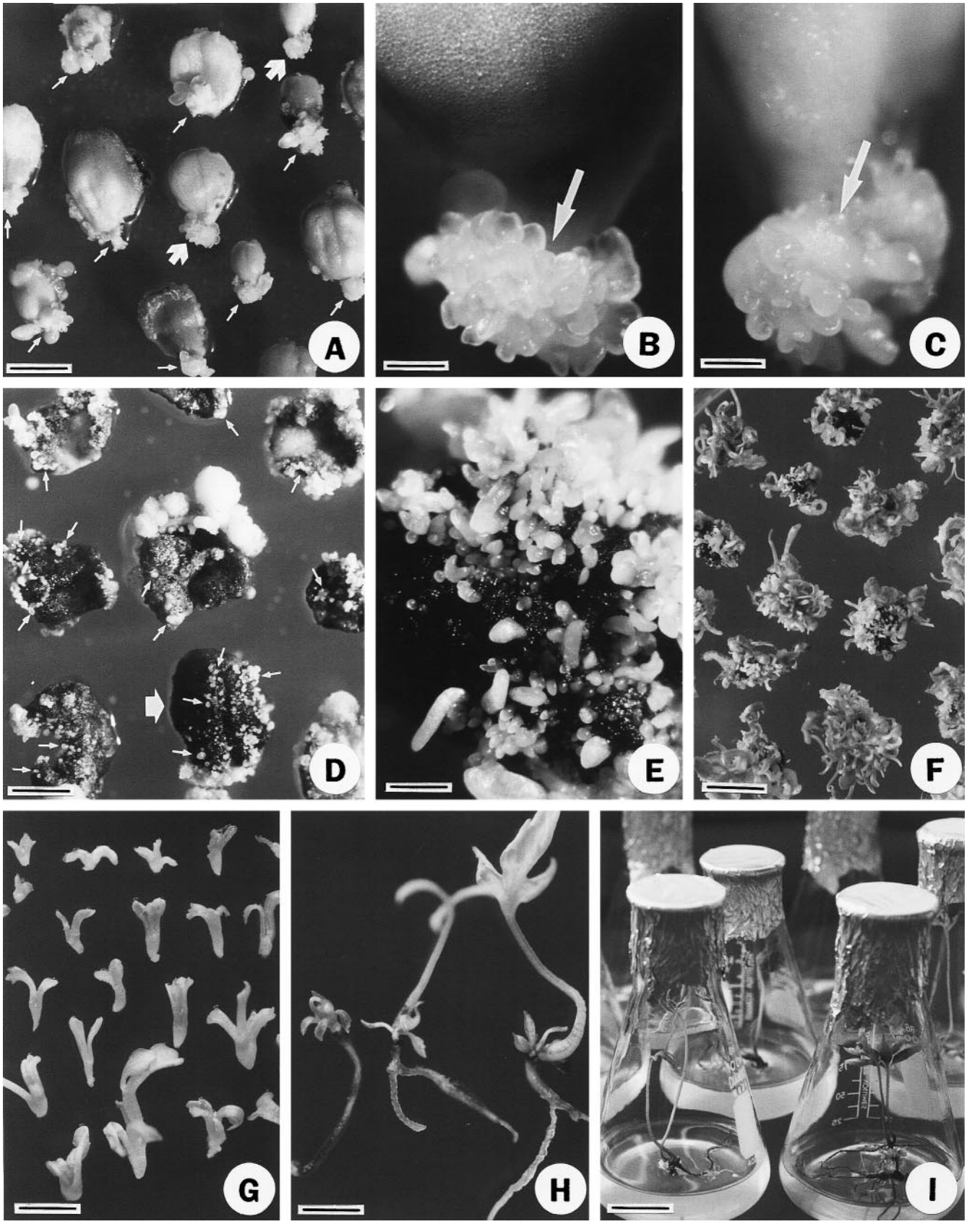
Results and discussion

High-frequency formation of single somatic embryos

When the cotyledon explants of immature ginseng zygotic embryos were cultured on MS medium lacking growth regulators, somatic embryos formed directly near the basal excised regions of cotyledon surfaces (Fig. 1A). Most of these somatic embryos developed in a multiple state, fused to each other and to the parent cotyledon explants (Figs. 1B, C, 2C). From histological observation, all the multiple somatic embryos were initiated from epidermal and subepidermal cell masses (Fig. 2A, B). When the cotyledon explants were pretreated with 1.0 M sucrose for 24–72 h, the frequency of single-embryo formation was markedly enhanced (Fig. 1D, E). With 72 h pretreatment, the number of somatic embryos per cotyledon rose to 36, about four times the control (Table 1), and these somatic embryos were formed from all surfaces of the cotyledon explants (Fig. 1D, E). Histological observation revealed that single somatic embryos formed from preplasmolyzed cotyledons developed from single epidermal cells over the whole cotyledon explant surface (Fig. 3A, B) and developed individually with a well-developed radicle (Fig. 3C–E).

Choi et al (1998) reported that somatic single embryos formed from germinating zygotic embryos regenerated into normal plants with both roots and shoots, but multiple embryos from immature zygotic embryos developed

Fig. 1A–G Somatic embryo formation from ginseng cotyledons with or without plasmolyzing pretreatment. **A–C** Multiple somatic embryos (*arrows*) formed directly from base of cotyledons without plasmolyzing pretreatment. **B–C** Enlarged view of **A** (*thick arrows*); note multiple embryos (*arrows*) fused to each other and to parent cotyledon explants (*bars* **A** 1 cm, **B, C** 1 mm). **D–I** High-frequncy single somatic embryogenesis and plant regeneration from preplasmolyzed ginseng cotyledons. **D** Numerous somatic single embryos (*arrows*) formed on all surfaces of cotyledon explants after plasmolyzing pretreatment with 1.0 M sucrose for 72 h (*bar* 7 mm). **E** Numerous single embryos on all surfaces of a cotyledon (*thick arrow* in **D**) at a later stage (after 2 months of culture) (*bar* 2 mm). **F** Germinating somatic single embryos formed on preplasmolyzed cotyledons by GA_3 treatments (*bar* 1 cm). **G** Somatic single embryos after separation from the cotyledon explants of **F** (*bar* 5 mm). **H** Small plantlets from single embryos with well-developed roots on 1/3 MS medium (*bar* 7 mm). **I** Plantlets grown on 1/3 MS medium in 100-ml Erlenmeyer flasks (*bar* 1.5 cm).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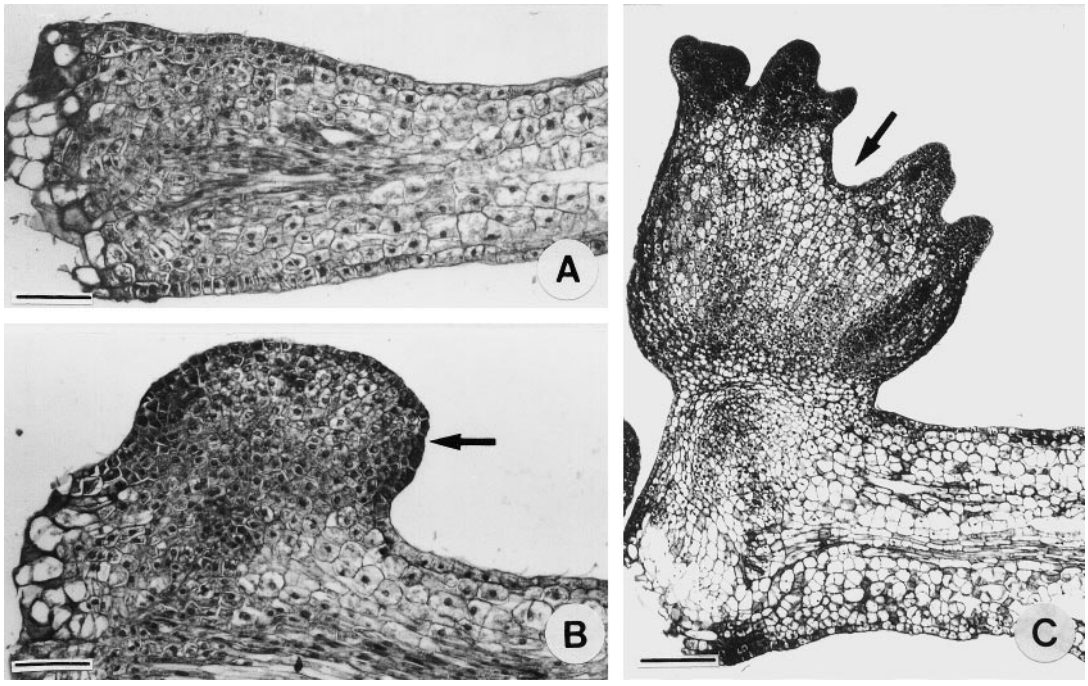


Fig. 2A–C Histological observation of multiple somatic embryo development from cotyledon explants of ginseng without plasmolyzing pretreatment. **A** Median longitudinal section of ginseng cotyledon just before culture (*bar* 80 μm). **B** An embryonic nodule (*arrow*) arising from the surface of the cotyledon base on hormone-free MS medium after 2 weeks of culture (*bar* 80 μm). **C** Multiple and fused somatic embryos (*arrow*) developing from a nodule after 1 month (*bar* 120 μm)

Table 1 Enhancement of direct somatic single-embryo formation (2 months after culture) from ginseng cotyledons, following plasmolyzing pretreatment (1 M sucrose). Data represent the mean values±SE of three independent experiments

Sucrose (1 M) pretreatment (h)	Number of somatic embryos/explant	Pattern of embryos (%)	
		Single	Multiple
No treatment	9±2.7	26	74
24	23±5.6	67	33
48	36±6.4	93	7
72	37±5.7	100	0

Table 2 Effect of chilling treatment on the germination of somatic embryos derived from cotyledon explants of *Panax ginseng*. Data represent the mean values±SE of three independent experiments. *No treatment* indicates that cotyledonary somatic embryos were cultured continuously temperature at 24°C

Chilling treatment (°C)	Germination after chilling treatment (%)			
	2 weeks	4 weeks	8 weeks	12 weeks
No treatment	0	0	0	0
4	0	0	5±1	8±2
0	0	6±3	12±3	13±4
–2	14±2	18±4	85±7	89±8

only into multiple shoots. Plasmolyzing pretreatment, therefore, can be a useful tool for both single-cell-derived somatic embryo formation and their normal plant regeneration in *P. ginseng*.

It is well known that plasmolysis of plant tissue physiologically isolates cells by disrupting the plasmodesmata which have a role in intercellular communication (Carr 1976). In lower plants such as algae, mosses, ferns and liverworts, stimulated regeneration from gemma or prothallia by plasmolysis has been achieved (Carr 1976). However, in higher plants, the effect of plasmolysis treatment on morphogenesis has only been reported in one other paper (Choi and Soh 1997).

Dormancy of somatic embryos

Maturation of somatic embryos normally proceeded until the cotyledonary stage on hormone-free MS medium, i.e., until 2 months of culture. The somatic embryos failed to germinate and regenerate plants, suggesting they were dormant. Even when transferred to fresh MS medium, the embryos remained white. There have been no reports on dormancy of somatic embryos of *P. ginseng*. However, in many papers, GA_3 treatment in combination of BAP or kinetin have often been used for maturation and shoot regeneration from ginseng somatic embryos (Chang and Hsing 1980; Shoyama et al. 1987; Arya et al. 1993). GA_3 breaks dormancy in many plant species (Bewley 1997). When somatic embryos of *P. ginseng* were treated with various concentrations (0–10 mg/l) of GA_3 greening and germination of somatic embryos were rapidly induced within 2 months of culture (Figs. 1F, 4). In seeds of *P. ginseng*, chilling treatment is commonly used to break the dormancy of zygotic embryos (Kuribayashi and Ohashi 1971). Somatic embryos of *P. ginseng* were treated with various low tem-

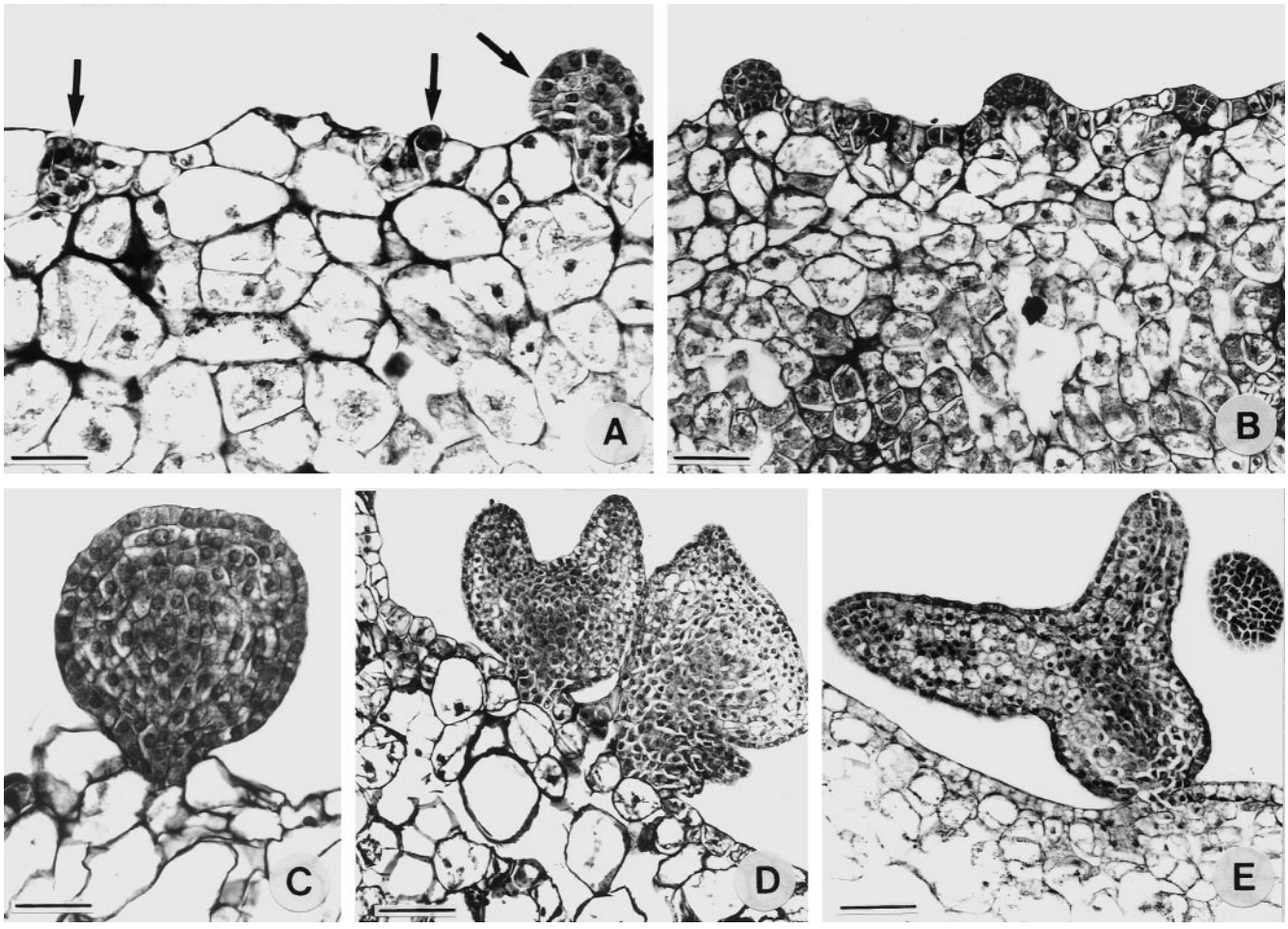


Fig. 3A–E Histological observations of somatic single embryos from single cells of the cotyledon epidermis after plasmolyzing pretreatment with 1.0 M sucrose for 72 h. **A**, **B** Numerous somatic embryos (*arrows*) arose directly from single epidermal cells of cotyledons after 2 weeks (*bars* **A** 60 μm, **B** 80 μm). **C** A single globular embryo after 1 month of culture (*bar* 60 μm). **D**, **E** Heart-shaped embryos (**D**) and a torpedo embryo (**E**) after 2 months of culture. Note that root poles were slightly attached to the cotyledon surfaces (*bars* 100 μm).

peratures (–2, 0, 4°C for 2–12 weeks) (Table 2). Eighty-five percent of somatic embryos germinated after 8 weeks treatment at –2°C; at temperatures above 0°C the somatic embryos did not germinate (Table 2).

Plant regeneration

After chilling and GA_3 treatment, numerous somatic single embryos (Fig. 1E) from preplasmolysed cotyledons turned green and covered the entire cotyledon surface but still did not produce roots (Fig. 1F). When these somatic embryos were manually removed individually from parent cotyledon explants by forceps (Fig. 1G) and transferred to 1/3-strength MS medium without growth regulators, about 87% of the embryos were regenerated into normal plants with both root and shoot after 2 months (Fig. 1H, I). However, multiple embryos formed from cotyledon explants without plasmolyzing pretreatment produced few roots and regenerated only multiple shoots (data not presented). Therefore, high-frequency plant production in ginseng cot-

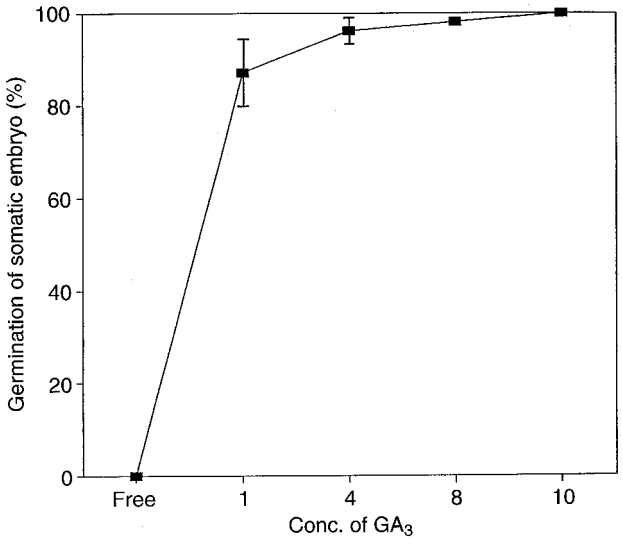
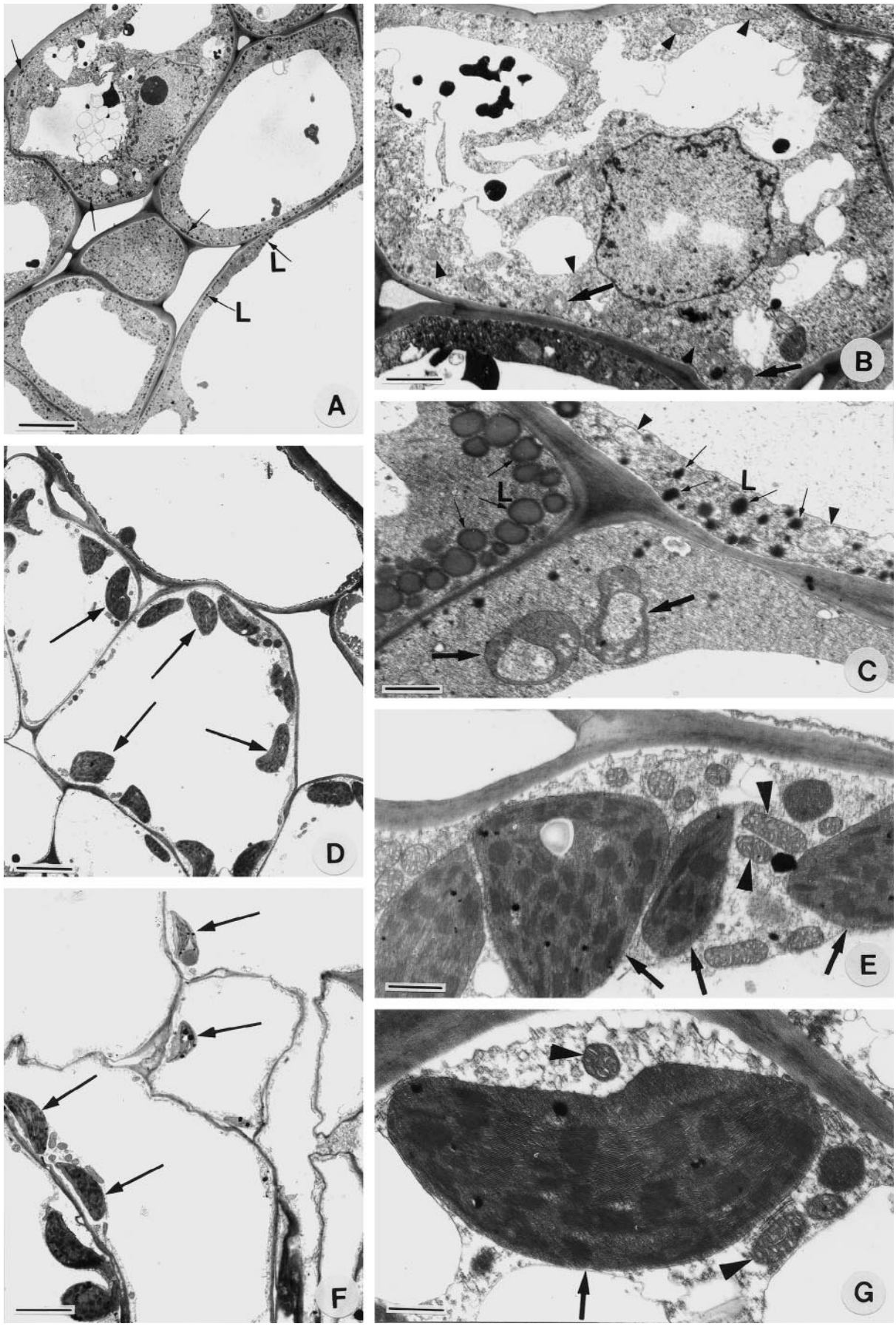


Fig. 4 Effect of GA_3 on germination of somatic embryos formed from cotyledon explants of *Panax ginseng*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yledon culture was accomplished by plasmolyzing pretreatment and a dormancy-breacking treatment.

Cell ultrastructure

Ultrastructural changes of cotyledon cells with or without GA_3 or chilling treatments were examined. Cotyledon cells of resting somatic embryos without GA_3 or chilling treatments contained numerous lipid bodies (Fig. 5A–C, thin arrows), dense cytoplasm, mitochondria with a poorly developed internal structure (Fig. 5B, C, arrowheads) and proplastids with poorly developed internal lamellae (Fig. 5B, C, thick arrows), similar to the cotyledon cells of dormant and resting zygotic embryos of *P. ginseng* (Choi et al. 1997). The cotyledon cells of somatic embryos after chilling treatments (–2°C for 8 weeks) did not contain lipid bodies, were highly vacuolated (Fig. 5D) and contained chloroplasts with membranous grana (Fig. 5E, arrow) and numerous mitochondria enclosing well-developed cristae (Fig. 5E, arrowheads). There are signs of metabolic activity active, similar to those seen in imbibed and germinating zygotic embryos of wheat (Swift and O'Brien 1972) and soybean (Webster and Leopold 1977). A similar phenomenon was observed in the cotyledon cells of somatic embryos before and after GA_3 treatment (Fig. 5F, G). Based on the above results, the rapid germination of somatic embryos following GA_3 and chilling treatments suggests they are dormant after maturing to the cotyledonary stage and require dormancy breaking treatment for germination. In the somatic embryos of *Vitis* (Takeno et al. 1983) and *Eschscholzia californica* (Kavathekar et al. 1977), GA_3 or chilling treatment were required for germination. However, in many plant tissue cultures, somatic embryos have a tendency to germinate prematurely without dormancy (Gray 1987). To induce quiescence or dormancy of somatic embryos, special treatments such as abscisic acid or dehydration treatments were required (Kitto and Janick 1985a, b; Gray 1987). Premature germination is not desirable for hardening of embryos, for production of synthetic seeds, and embryo conservation. Therefore, the dormant state of somatic embryos of *P. ginseng* might have some advantages for the germination of uniform somatic embryos and synthetic seed production.

In conclusion, mass production of ginseng plants was accomplished via enhanced direct single embryogenesis from preplasmolysed ginseng cotyledons, and ginseng somatic embryos had dormant characters similar to their zygotic counterparts.

Fig. 5A–G Ultrastructural changes of cotyledon cells of somatic embryos with or without chilling or GA_3 treatment (*bars* **A**, **D**, **F** 6 μm, **B** 3 μm, **C**, **E**, **G** 1 μm). **A–C** Cotyledon cells of somatic embryos without chilling and GA_3 treatment. Note dense cytoplasm, numerous lipid bodies (*L*, *thin arrows*), proplastids (*thick arrows* in **B**, **C**) and poorly differentiated mitochondria (*arrowheads* in **B**, **C**). **D**, **E** Cotyledon cells after chilling treatment (–2°C for 8 weeks). Note well-developed chloroplasts (*arrows*) and active-state mitochondria with numerous cristae (*arrowheads* in **E**). **F**, **G** Cotyledon cells after 8 mg/l GA_3 treatment. Note similar cellular structure to **D**, **E**

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