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ORIGINAL ARTICLE

Plant regeneration via direct somatic embryogenesis in *Panax japonicus*

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Abstract *Panax japonicus* is one of the important medicinal plants. Here, we established the protocol for plant regeneration of *P. japonicus* via direct somatic embryogenesis. Somatic embryos were directly obtained from the segments of zygotic embryos on MS medium with 4.4 μM 2,4-D. Thereafter, somatic embryos were produced by repetitive secondary somatic embryogenesis. The secondary somatic embryo formation was enhanced by plasmolyzing pretreatment (1.0 M mannitol for 10 h). Frequency of secondary somatic embryo formation from cotyledon segments was lowered by plasmolyzing pretreatment, but the number of somatic embryos per explants was greatly increased. Plasmolyzing pretreatment resulted in retardation of embryo growth and required subculture to fresh medium for further growth of embryos into cotyledonary stage. Without plasmolyzing pretreatment, cotyledonary embryos were obtained after 8 weeks of culture. All the cotyledonary somatic embryos germinated by 5 μM GA_3 treatment, but only 15.3% were germinated on hormone-free medium. After 2 months of culture on 1/2 strength WPM medium, plantlets produced flowers spontaneously. In the anthers of in vitro flowers, microsporogenesis occurred normally with low number of pollen grains.

Keywords Embryogenesis · Medicinal plants · *Panax japonicus* · Plasmolyzing pretreatment

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Introduction

Genus *Panax*, a family Araliaceae, comprises several species of slow-growing perennial herbaceous plants with thickened roots. They grow in the Northern Hemisphere in eastern Asia (mostly Korea, northern China, and eastern Siberia) and North America, typically in cooler climates. Roots of *P. ginseng* are characterized by the presence of pharmacologically active triterpene called ginsenosides. The ginsenosides have been shown to have a variety of beneficial effects, including anti-inflammatory, antioxidant, and anticancer effects (Shibata 2001; Vogler et al. 1999).

The rhizomes of *P. japonicus* C.A. Meyer have been used as a substitute for *P. ginseng* C.A. Meyer. It has been reported that the rhizomes of *P. japonicus* have anti-ulcer action and fibrinolysis (Yamahara et al. 1987; Matsuda et al. 1989) and have been used to sooth coughs and reduce phlegm (Chang and But 1986).

Because of the high medicinal values of *Panax* species, there has been continuous harvest of these plants over thousands of years and the natural sources of these species have become almost extinct and are under threatened or endangered status (Tran et al. 2003; Stokstad 2005). In addition, ginseng plants do not set seeds until after about 4 years and the number of seeds is very small. Micropropagation of *Panax* species is a valuable method for conservation and propagation of these species. In *P. ginseng*, plant regeneration via somatic embryogenesis has been extensively reported (Chang and Hsing 1980a; Choi et al. 1998). In *P. japonicus*, plant regeneration by somatic embryogenesis from flower bud and rhizome was reported by Fujioka et al. (1986). However, the frequency of plant regeneration remains to be improved.

The aim of the present study was to focus on high frequency plant regeneration via direct somatic embryogenesis by plasmolyzing pretreatment and in vitro flower formation in *P. japonicus*.

Materials and methods

Plant materials

Seeds of wild *P. japonicus* C.A. Meyer were collected from Nikko National Mountain in Japan. They were stratified in moist sand until the zygotic embryo became mature. Then, after removing the coat, the seeds were sterilized in 70% ethyl alcohol for 1 min and 2% of NaOCl for 20 min, and rinsed three times with sterile water. Zygotic embryos were dissected out and used for initial culture materials.

Induction of somatic embryo from zygotic embryos

Cotyledons, hypocotyls and radicles of pre-germinated zygotic embryos and 2-week-old post-germinated zygotic embryos were cultured on MS (Murashige and Skoog 1962) solid (0.8% agar) medium with 3% sucrose and 4.4 μM 2,4-D. Frequency of somatic embryos was examined after 8 weeks of culture. The medium was adjusted to 5.8 pH before autoclaving at 120°C for 15 min. The culture room was maintained at 24 ± 2°C with a 16:8 h (day:night) photoperiod of 24 μmol m^{-2} s^{-1} under cool white fluorescent tubes. Thirty explants were cultured in petri dishes. Each experiment was performed three times.

Direct secondary somatic embryo induction

Cotyledonary somatic embryos were immersed in 1.0 M mannitol for 0, 5, 10 and 20 h to induce plasmolysis. Deplasmolysis was carried out by gradually decreasing the concentration of mannitol from 1.0 to 0.125 M. Deplasmolysed zygotic embryos were cultured on MS medium with 2.2 μM 2,4-D in 10 × 1.5 cm petri dishes containing 30 ml medium. Thirty explants were cultured in petri dish. Each experiment was performed three times. After 8 weeks of culture, frequency and number of somatic embryos were noted.

Germination and conversion into plantlets

Cotyledonary somatic embryos were transferred onto MS medium with or without 5 μM GA_3. Frequency of somatic embryo germination was examined after 5 weeks of culture. After germination, small plantlets with shoots and roots were cultured on 1/2 WPM (McCown and Lloyd 1981) medium (2% sucrose) to support their growth. After 2 months, growth of plant was investigated.

Results and discussion

Somatic embryo induction from zygotic embryos

Excised cotyledons, hypocotyls and radicles from two stages (pre- and post germination) of zygotic embryos were cultured on MS medium with 4.4 μM 2,4-D for 8 weeks. Among the cultured explants (cotyledon, hypocotyl and radicle), frequency of somatic embryo formation was higher in explants of pre-germinated zygotic embryos than post-germinated ones (Fig. 1). Cotyledon explants were more effective for somatic embryo formation compared to hypocotyl explants and radicle (Fig. 1). Most of somatic embryos were developed directly close to the excised margin of explants and matured to cotyledonary stage after 8 weeks of culture (Fig. 2a). Embryogenic callus were also produced at a frequency of <12% and developed somatic embryo on hormone-free MS medium (data not shown).

Direct somatic embryos induced from the cotyledonary somatic embryo

Cotyledonary somatic embryos pre-plasmolyzed with 1.0 M mannitol for 0, 5,10 and 20 h were cultured on MS medium with 2.2 μM 2,4-D. After 8 weeks, numerous somatic embryos at various developmental

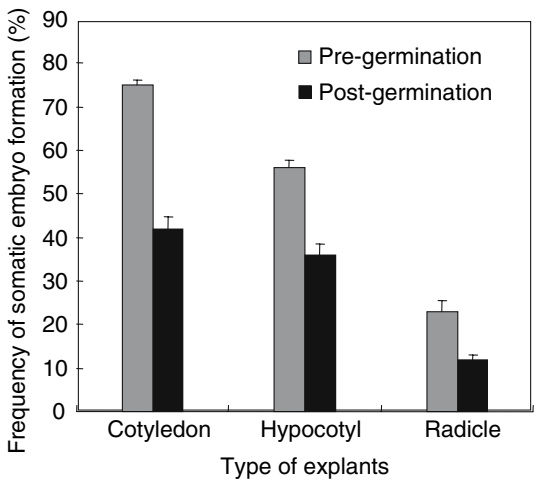

Fig. 1 Frequency of somatic embryo formation from different stages of zygotic embryos of *Panax japonicus* on MS medium with 4.4 μM 2,4-D after 8 weeks of culture

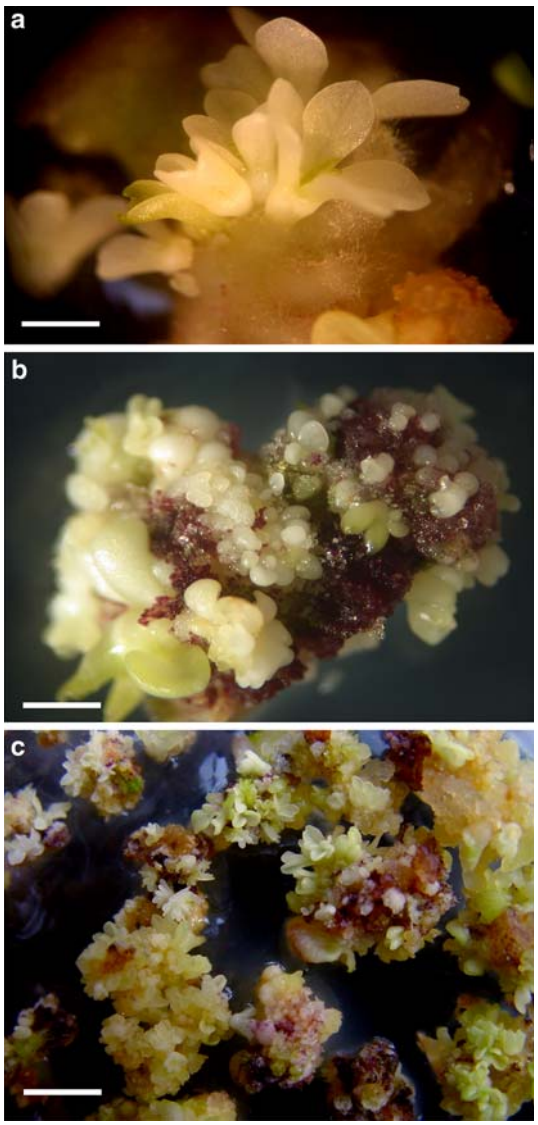

Fig. 2 Somatic embryo formation from the hypocotyl of somatic embryos on MS medium with 2.2 μM 2,4-D after plasmolyzing pretreatment with 1.0 M mannitol for 24 h. **a** Somatic embryo formation directly from hypocotyl explants of zygotic embryos without plasmolyzing pretreatment (*bar* = 1.5 mm). **b** Direct secondary somatic embryo formation from cotyledon explants of somatic embryos after plasmolyzing pretreatment for 10 h (*bar* = 1.5 mm). **c** Low magnification view of somatic embryo formation after plasmolyzing pretreatment for 10 h (*bar* = 3.5 mm)

stages were formed from the surfaces of explants (Fig. 2a). Frequency of somatic embryos was decreased by plasmolyzing pretreatment (Fig. 3a). However, among the different plasmolyzing pre-treatment times, 10-h pretreatment markedly increased the number of somatic embryos (above 60.6 embryos per explant) compared to other treatments (Fig. 3b). Growth of somatic embryos was retarded by longer plasmolyzing pretreatment (Fig. 3c). Thus, the ratio of somatic embryo stages was different among the different plasmolysis treatments (Fig. 3d). Without pre-plasmolyzing pretreatments, the average 51.6% of somatic embryos was less than torpedo stage after 8 weeks of culture (Figs. 2b, 3d), whereas 51.2% of somatic embryos remained at globular or heart-shaped stage after 20 h of plasmolyzing pretreatment (Fig. 2c, 3d). To stimulate the maturation of these somatic embryos, transferring them into fresh hormone-free MS medium was necessary. This result indicates that plasmolysis pretreatment stimulates the synchronized development of somatic embryos but growth of somatic embryos is retarded. The retarded growth of somatic embryos might be caused by the single celled-derived somatic embryogenesis. Thus, to induce the highest number and synchronized development of somatic embryos, the best treatment time for plasmolyzing pretreatment was 10 h. Choi and Soh (1997) reported that 1.0 M sucrose pretreatment of cotyledon segments of *P. ginseng* for 24 h induced a high frequency of single cell-derived somatic embryos. In zygotic embryos of *Eleutherococcus senticosus*, plasmolyzing pretreatment strongly enhanced the frequency of direct somatic embryos (You et al. 2006). You et al. (2006) reported that enhanced single cell-derived somatic embryogenesis directly from the surface of explants coincided with the rapid accumulation of callose in the cell wall due to plasmolyzing pretreatment. In *Pelvetia* embryo culture, transient plasmolysis of rhizoid cells resulted in abandonment of pre-existing apex and the initiation of a new rhizoid tip after rehydration (Kropf et al. 1993).

Germination of somatic embryos and conversion into plantlets

When explants comprising various stages of somatic embryos were transferred onto fresh medium lacking 2,4-D, growth of somatic embryos was stimulated until the cotyledonary stage but did not proceed into germination. Somatic embryos were rapidly germinated when they were transferred onto MS medium containing 5 μM GA_3 (Fig. 4a). All the embryos (100%) turned green and germinated after 5 weeks of culture (Table 1), while most somatic embryos remained at yellow-white color without germination on GA_3-free MS medium. Without GA_3 treatment, secondary somatic embryos were developed directly on the surfaces of somatic embryos (Table 1). Stimulation of germination of somatic embryos by GA_3 treatment has beenreported in *Panax ginseng* by Choi et al. (1999) and *Eleutherococcus senticosus* (Choi et al. 1999). Choi et al. (1999) interpreted the requirement of GA_3 for

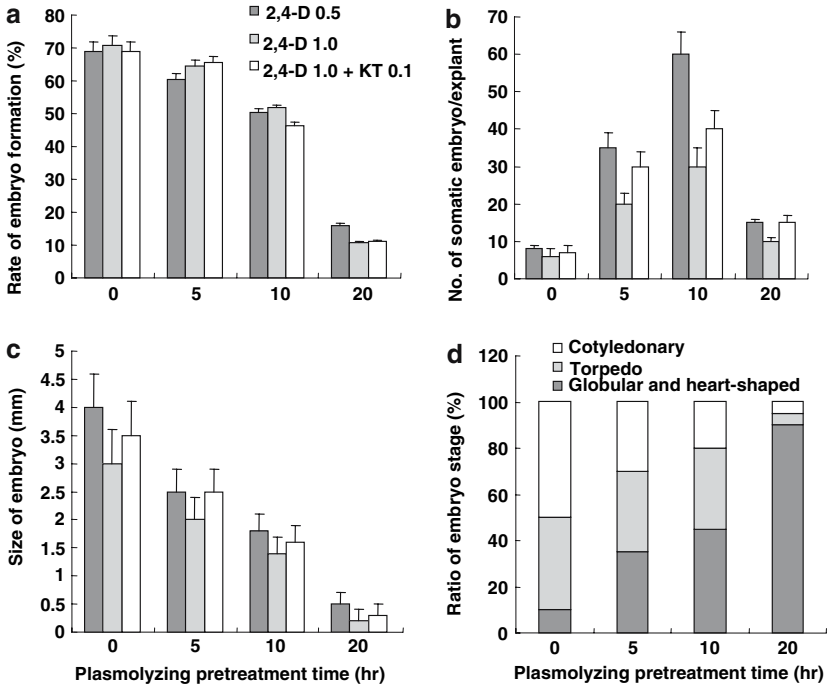

Fig. 3 Frequency (**a**) of somatic embryo formation, number (**b**) of somatic embryos per explants, size (**c**) of somatic embryos and ratio of different stages of somatic embryos (**d**) after plasmolyzing pretreatment with 1.0 M mannitol for 0, 5, 10 and 20 h

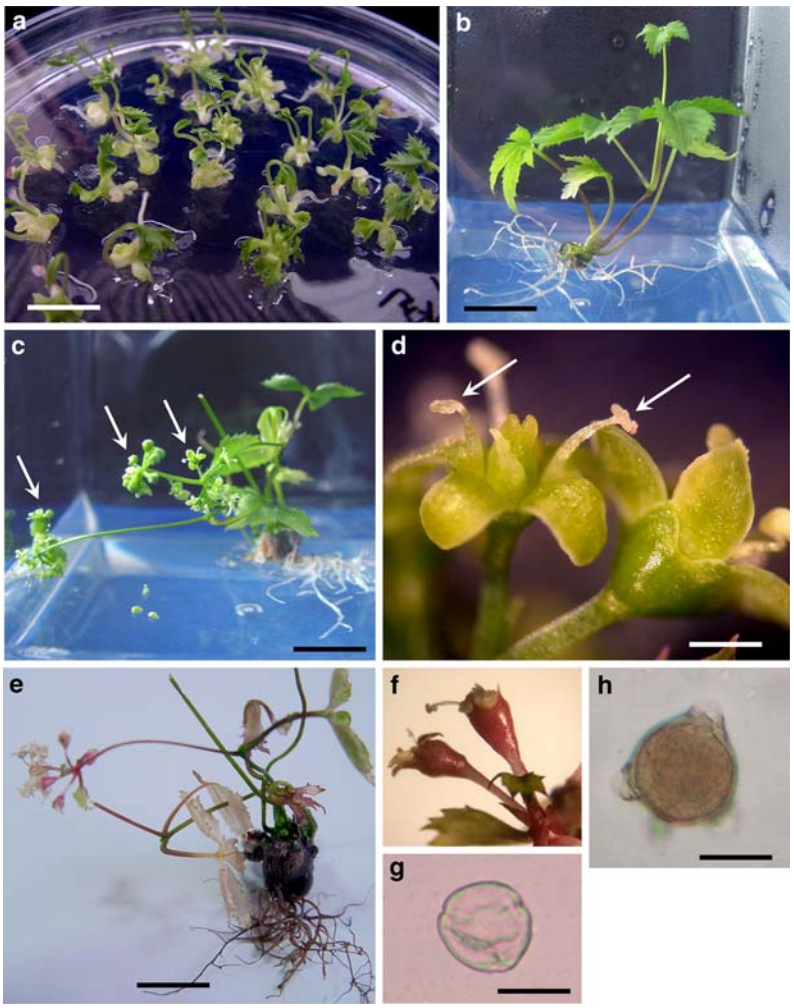

Fig. 4 Conversion of somatic embryos into plantlets and in vitro flower formation. **a** Germination of somatic embryos on MS medium with 5 μM GA_3 after 2 weeks of culture (*bar* = 10 mm). **b** Plantlet grown on hormone-free 1/2 WPM medium after 1 month of culture (*bar* = 10 mm). **c** In vitro flowering of plantlets (*arrows* indicate flowers) (*bar* = 10 mm). **d** Closed view of flower (*arrows* indicate anthers) (*bar* = 3 mm). **e** Colored (senescence) flowers with thickened ovary (*bar* = 10 mm). **f** Closed view of flowers shown in **e**. **g** Microspore from the anther of green flower buds shown in **c** (*bar* = 100 μm). **h** Mature pollen isolated from the anther of colored flower shown in **e** (*bar* = 100 μm)

Table 1 Germination of somatic embryos of *Panax japonicus* on the MS medium with and without GA_3 after 5 weeks of culture

Treatment	Germination rate (%)	Frequency of secondary somatic embryo formation
Sucrose 3%	15.3 ± 1.7	22.6 ± 2.7
Sucrose 5%	7.2 ± 1.3	37.5 ± 2.3
Sucrose 3% + 5 μM GA_3	100	–

the germination of somatic embryos as due to the dormancy of somatic embryos. In zygotic embryos of the Araliaceae family, seeds have double dormancy: morphological dormancy (rudimentary embryos just after harvest of fully matured seed) and physiological dormancy after maturity, requiring cold treatment for 3 months (Isoda and Shoji 1989).

In vitro flowering of plantlets

Germinated somatic embryos were transferred onto 1/2 WPM medium with 2% sucrose. They were grown into plantlets after 2 months of culture (Fig. 4b). Continuous culture of plantlets on 1/2 WPM medium with 2% sucrose resulted in flowering spontaneously in vitro (Fig. 4b). Frequency of in vitro flowering of plantlets was 45.7% even without special treatment. This result indicates that in vitro plantlets of *P. japonicus* are capable of producing in vitro flowers. In *P. ginseng*, combined treatment of both cytokinin and GA_3 was necessary to induce in vitro flowers (Chang and Hsing 1980b). Flowers of in vitro plantlets were smaller than that of wild *ginseng* but showed normal structure with well-developed petals, pistils and stamens (Fig. 4c–f). Microspores in the anthers were very low in number (about 200 per anther), but some microspores were fully matured having normal morphology with a well-developed pollen wall (Fig. 4 g–h).

In conclusion, we established plant regeneration via direct somatic embryogenesis in *P. japonicus* and the formation of in vitro flowers, which can be used for rapid breeding by crossing to field cultivated plants, if their pollens are functionally normal, and genetically transformed plants.

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References

Chang H, But P (1986) Pharmacology and application of Chinese material medica. vol 1. World Scientific, Singapore, pp 17

Chang WC, Hsing YI (1980a) Plant regeneration through somatic embryogenesis in root-derived callus of *ginseng* (*Panax ginseng* C. A. Meyer). Theor Appl Genet 57:133–135

Chang WC, Hsing YI (1980b) In vitro flowering of embryoids derived from mature root callus of *ginseng* (*Panax ginseng*). Nature 284:341–342

Choi YE, Soh WY (1997) Enhance somatic single embryo formation by plasmolyzing pretreatment from cultured *ginseng* cotyledons. Plant Sci 135:197–206

Choi YE, Yang DC, Par JC, Soh WY, Choi KT (1998) Regeneration ability of somatic single and multiple embryos arising directly from cotyledons of *Panax ginseng*. Plant Cell Rep 17:544–551

Choi YE, Kim JW, Yoon ES (1999) High frequency of plant production via somatic embryogenesis from callus or cell suspension cultures in *Eleutherococcus senticosus.* Ann Bot 83:309–314

Fujioka N, Kurisu Y, Miyagawa H, Kohda H (1986) Studies on the tissue culture of *Panax japonicus* (1) Multiplication by somatic embryogenesis of flower bud and rhizome. Shoyakugaku Zasshi 40:152–158

Isoda S, Shoji J (1989) Studies on the cultivation of *Eleutherococcus senticosus* maxim. I On the after-ripening and breaking dormancy. Syoyakugaku Zasshi 43:70–88

Kropf DL, Coffman HR, Kloareg B, Glenn P, Allen VW (1993) Cell wall and rhizoid polarity in *Pelvetia* embryos. Dev Biol 160:303–314

Matsuda H, Samukawa K, Fukuda S, Shiomoto H, Tung CN, Kubo M. (1989) Studies of *Panax japonicus* fibrinolysis. Planta Med 55:18–21

McCown BH, Lloyd G (1981) Woody plant medium (WPM)—a mineral nutrient formulation for microculture for woody plant species. Hort Sci 16:453

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

Shibata S (2001) Chemistry and cancer preventing activities of *ginseng* saponins and some related triterpenoid compounds. J Korean Med Sci 16:S28–37

Stokstad E (2005) *Ginseng* threatened by Bambi's appetite. Science 307:827

Tran QL, Than MM, Tezuka Y, Banskota AH, Kouda K, Watanabe H, Zhu S, Komatsu K, Thet MM, Swe T, Maruyama Y, Kadota S (2003) Wild *ginseng* grows in Myanmar. Chem Pharm Bull 51:679–682

Vogler BK, Pittler MH, Ernst E (1999) The efficacy of *ginseng*. A systematic review of randomised clinical trials. Eur J Clin Pharmacol 55:567–575

Yamahara J, Kubomura Y, Miki K, Fujimura H. (1987) Anti-ulcer action of *Panax japonicus* rhizome. J Ethnopharmacol 19:95–101

You XL, Yi JS, Choi YE (2006) Cellular changes and callose accumulation of zygotic embryos of *Eleutherococcus senticosus* by plasmolyzing pretreatment result in high frequency single cell-derived somatic embryogenesis. Protoplasma 227:105–112