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Dormancy of Somatic Embryos Derived from the Cotyledon of Korean Ginseng

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Abstract : Somatic embryos were induced directly from cotyledon explants of Korean ginseng (*Panax ginseng* C.A. Meyer) on Murashige and Skoog (MS) medium with 2,4-D, BAP, kinetin or lacking growth regulators. When somatic embryos formed on all media grew to cotyledonary stage, the further development of embryos was ceased and remained in white color. By gibberellic acid (over 1.0 mg/l GA_3) treatment, all the somatic embryos turned rapidly to green and germinated within 3 weeks. Chilling treatment also induced the germination of somatic embryos. The effective temperature regime was -2°C for over 8 weeks but more higher temperature than 0°C did not effective for 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 with poorly differentiated internal structure, but the cotyledon cells of germinating somatic embryos after chilling or GA_3 treatment highly vacuolated and contained well-developed chloroplasts and active state of mitochondria enclosing numerous cristae. The above results indicate that *in vitro* developed somatic embryos of *Panax ginseng* may be dormant after mature similar to zygotic embryos.

Key words : Dormant somatic embryo, chilling treatment, GA_3 treatment, germination, *Panax ginseng*.

Introduction

In general, the growth of zygotic embryos in the plant seeds was ceased after mature, and eventually dormant or quiescent.[1] The resting phase of zygotic embryos greatly improve the conservation ability of seeds for long duration and has been described as an adaptive trait that the seeds germinate at a favourable time for survival of seedlings.[2]

Somatic embryos formed from plant tissue cultures are similar to zygotic embryos physiologically and morphologically.[3] In contrast to zygotic embryos, it has been reported that somatic embryos have a tendency of premature germination without dormancy when dormancy inducing treatments are not given.[3,4] The premature germination is not desirable for hardening of embryos, for production of synthetic seeds and conservation of embryos. Therefore, to induce quiescence or dormancy of somatic embryos, special treatments such as ABA or dehydration treatment were required.[4-6] Exceptionally, somatic embryos derived from some plant species such as *Vitis*,[7] *Eschscholzia californica*[8] require chilling treatment similar to zygotic embryos.

Zygotic seeds of *Panax ginseng* are required the chilling treatment for about 3 months for dormancy breaking.[9] In somatic embryos of *Panax ginseng*, the combination of cytokinin (BAP) and GA_3 highly efficient for the plant development from somatic embryos.[10-12] However, it is not known of whether the somatic embryos of *Panax ginseng* have dormant characteristic similar to the zygotic embryos in the seeds.

The present study deals with the effect of chilling and GA_3 treatment on the germination and plant regeneration from somatic embryos and discussed the possible dormancy of somatic embryos of *Panax ginseng*.

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Materials and Methods

1. Somatic embryogenesis

Korean ginseng (*Panax ginseng* C.A. Meyer) seeds were harvested from experimental garden of the Korea Ginseng and Tobacco Research Institute. The seeds were stratified in humidified sand for 3 months and then stored at a 4°C temperature for further 3 months since the zygotic embryos just after harvest were in a mature cotyledonary stage (at about 500 μm in length). The seeds were immersed in 70% ethanol for 1 min, then in 1% sodium hypochlorite solution for 1 h, and washed 3 times with sterile distilled water. After carefully dissecting the zygotic embryos from the seeds, the abaxial sides of the excised cotyledons were placed on the surface of Murashige and Skoog[13] basal medium with 3% sucrose, 0.7% agar and with auxin (1.0 mg/*l* 2,4-D), cytokinins (1.0 mg/*l* BAP or 1.0 mg/*l* kinetin) or lacking growth regulators. The medium was adjusted to pH 5.8 and then was autoclaved at 120°C for 15 min. Cotyledon explants were cultured in 10×2 cm plastic petri dishes containing 30 m*l* of medium. Fifteen cotyledon explants were cultured on each petri dish. Three replicates were used for per treatment which was repeated 3 times. The culture room was maintained at 24±2°C under a 16-8 h photoperiod of 24 μmol $m^{-2}s^{-1}$ intensity under white fluorescent tubes. The frequency of somatic embryo production was determined after 2 months of culture by counting the number of cotyledon explants forming somatic embryos.

2. Gibberellic acid treatment

To investigate the effect of GA_3 on the germination of somatic embryos, somatic embryos were transferred to MS medium with various concentrations of GA_3 (0, 1, 5, 10, 20 mg/*l*). The medium contained MS basal medium with 3% sucrose and 0.7% agar. After 2 months of culture, the germination of somatic embryos was assessed. Somatic embryos were cultured on 10×2 cm petri dish. About 20 explants were used per treatment, which was repeated 3 times.

3. Chilling treatment

To investigate the effect of chilling treatment on the germination of somatic embryos, the petri dishes contained somatic embryos were transferred to low temperature incubator for 4 to 12 weeks. Temperature regimes were adjusted to various levels (-2, 0, 4, 25°C). After those treatment, the petri dishes containing somatic embryos were transferred to incubation room. After 2 months of culture, the germination of somatic embryos was assessed.

4. Ultrastructural observation

To observe histologically the cellular events after cold and GA_3 treatment, cotyledon segments of 1 mm^3 were sampled from the resting somatic embryos without treatment and from somatic embryos after chilling treatment at -2°C (8 weeks) or GA_3 treatment (10 mg/*l* for 1 month). Sampled cotyledon tissues were fixed in 1% glutaraldehyde (phosphate buffer pH 6.8) for 4 h at 4°C and post fixed in 2% OsO_4 for 2 h. The samples were dehydrated with ethyl alcohol and embedded in Epon regins. The ultrathin sections were made from median longitudinal sections of cotyledons using LKB-V ultramicrotome. Thin sections were stained with 1% uranyl acetate and lead citrate. The sections were observed by trans-mission electron microscope (JEM 1200 EX-).

Results and Discussion

1. Somatic embryo formation

When the cotyledon explants of *P. ginseng* were cultured on MS medium lacking growth regulators, somatic embryos developed directly near the basal excised portion of cotyledons (Fig. 1A). The growth of somatic embryos was normally proceed until cotyledonary stage on MS medium without special treatment for 2 months of culture. However, the further development of somatic embryos such as germination and plant regeneration did not proceed further. Even though somatic embryos were transferred to fresh medium, the embryos did not germinate but stayed in white color, or they produced secondary somatic embryos after some abnormal expansion of somatic embryos (Fig. 1B). In somatic embryos formed directly or indirectly from the cotyledon explants on medium with auxin (1.0 mg/*l* 2,4-D), the embryos also did not germinate and stayed in white color after mature to cotyledonary stage. Fig. 1C showed that callus-derived somatic embryos on medium with 1.0 mg/*l* 2,4-D were stayed in white color without senescence until 10 months but the other callus was browned darkly. A similar dormant phenomena of somatic embryos of *P. ginseng* also observed in the embryos formed on

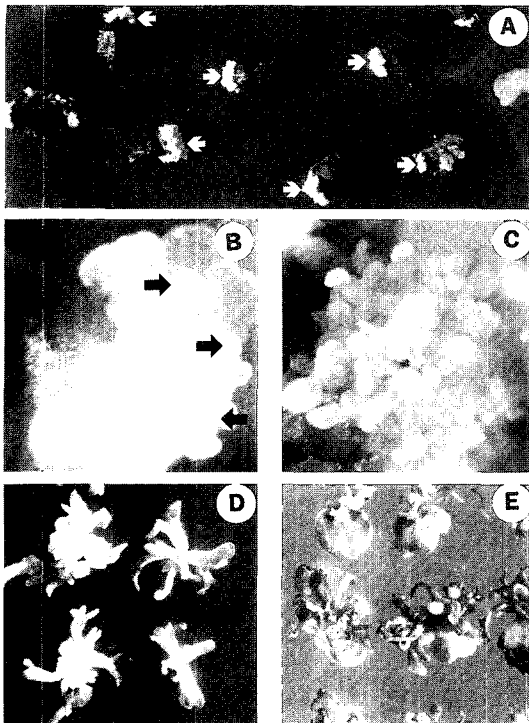

Fig. 1. A. Cotyledonary somatic embryos (arrows) formed from cotyledon explants of *P. ginseng* on MS medium lacking growth regulators after 2 months, notes no germination of embryos with white in color. B. Cotyledonary somatic embryos after separation from maternal cotyledon explants showing no germination but producing secondary embryos (arrows) from abnormally expanded cotyledons on fresh MS basal medium. C. Ten month-old cotyledonary somatic embryos derived from callus of cotyledon explants on MS medium with 1.0 mg/*l* 2,4-D, notes darkly browned callus except for somatic embryos in white color without senescence. D. Germination and plant regeneration of somatic embryos on the medium with 10 mg/*l* GA_3. E. Germination and plant regeneration from somatic embryos after chilling treatment (-2°C for 8 weeks).

MS medium with 1.0 mg/*l* BAP or 1.0 mg/*l* kinetin (data not presented).

2. GA_3 treatment

All the cotyledonay somatic embryos formed on primary medium were not germinated and retained in white color. The sole GA_3 treatment at over 1.0 mg/*l* rapidly induced the greening within 3 weeks of culture, and single somatic embryos induced on MS medium lacking growth regulator were germinated (Fig. 1D, Table 1). Cytokinin (1 mg/*l* BAP or 1 mg/*l* kinetin)

Table 1. Effect of growth regulators on the germination of somatic embryos derived from cotyledon explants of *Panax ginseng*

Concentration of growth regulators (mg/*l*)		Germination ratio (%)
GA_3 .	0	0[a]
	1	87±12
	5	93±8.2
	10	98±6.5
	20	98±3.4
BAP	1	12±2.4
kinetin	1	14±4.9

[a]Data represent the mean values±SE of three independent experiments.

treatment did not efficient for the germination of ginseng somatic embryos (Table 1). In general, gibberellic acid is the well known substance for dormancy breaking in many plant species[1,2]. There have been no report on the dormancy of somatic embryos of *P. ginseng*. However, to induce the maturation and shoot regeneration from somatic embryos of *P. ginseng*, GA_3 treatment in combination of BAP or kinetin was commonly used[10,11,12] and these media were also used for the flower bud induction from axialary buds in the cotyledonary nodes of ginseng zygotic embryos or somatic embryos.[14]

3. Chilling treatment

Generally chilling treatment can replace the GA_3 treatment for dormancy breaking of seeds[8]. In seeds of *Panax ginseng*, chilling treatment is commonly used to break the dormancy of zygotic embryos.[9] When somatic embryos of *P. ginseng* were treated with various low temperatures (-2, 0, 4, 25 for 4 to 12 weeks) as represented in Table 2. By the low temperature treatment, 85% of somatic embryos induced on medium with 2,4-D and germinated and regenerated into plants after 8 weeks treatment at -2 (Fig. 1E, Table 2). However, more higher temperature at over 0 was not effective for germination of somatic embryos. Based on the above results, the rapid responce of somatic embryo germination by GA_3 or chilling treatments might represent that somatic embryos of *P. ginseng* are probably dormant after mature to cotyledonary stage.

4. Ulturastructure of cotyledon cells

Cotyledon cells of somatic embryos before and after chilling treatment (8 weeks at -2) and GA_3 treatment (2 weeks at 10 mg/*l*) were observed ultrastructurally (Fig.

Table 2. Effect of chilling treatment on the germination of somatic embryos derived from cotyledon explants of *Panax ginseng*

Chilling treatment (°C)	Germination after chilling treatment (%)		
	4	8	12 (week)
-2	18±4[a]	85±7	89±8
0	6±3	12±3	13±4
4	0	5±1	8±2
25	0	0	0

[a]Data represent the mean valuesSE of three independent experiments.

2). In the resting somatic embryos with no treatment, cotyledon cells contained numerous lipid bodies, dense cytoplasm, mitochondria with a poorly developed internal structure without cristae and proplastids with poorly developed internal lamellae (Fig. 2A,B), which is similar to the cotyledon cells of dormant zygotic embryos of *P. ginseng*.[15] In the somatic embryos after GA_3 and chilling treatments, the cotyledon cells in both treatments did not contain lipid bodies, highly vacuolated and contained chloroplasts with the membranes of grana and numerous mitochondria enclosing well-developed cristae (Fig. 2C, D). The well-developed chloroplasts and active state of mitochondria in the cotyledon cells after GA_3 or chilling treatment represent the metabolically active state, which is similar to that of the imbibed and germinating zygotic embryos in wheat and soybean.[16,17]

Similar to the somatic embryos of *P. ginseng*, somatic embryos of *Vitis*[7] and of *Eschscholzia californica*[8] required GA_3 or chilling treatment for germination. However, the dormancy of somatic embryos is not common event in the all species. In the zygotic embryos of *Acanthopanax senticosus* and *A. koranum*, same family to *P. ginseng* (Araliaceae), chilling treatment was essential for germination of seeds.[18] However, somatic embryos of these species were germinated normally without any special treatment.[15,19] Generally, somatic embryos in many plants rather have a tendency of premature germination.[3] The premature germination is not desirable for hardening of embryos and synthetic seed production. Therefore, the dormant state of somatic embryos of *P. ginseng* might have some advantages on the uniform germination of somatic embryos and synthetic seed production.

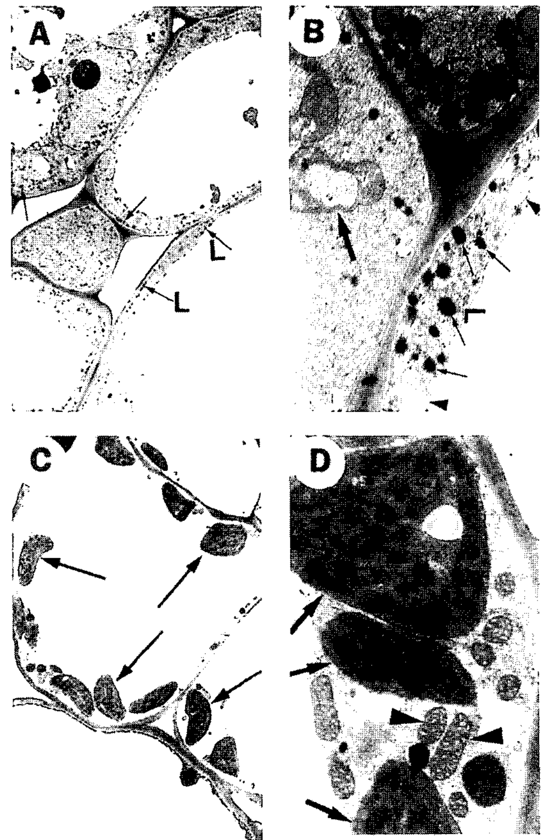

Fig. 2. Transmission electron micrograph of cotyledon cells of somatic embryos with or without chilling and GA_3 treatment. A,B. Cotyledon cells of somatic embryos without chilling and GA_3 treatment, notes dense cytoplasm and numerous lipid bodies (L, thin arrows), proplastids (thick arrows) and mitochondria (arrowheads). C,D. Cotyledon cells after chilling treatment (-2°C for 8 weeks) and after 10 mg/*l* GA_3 treatment, notes well-developed chloroplasts (arrows) and active state of mitochondria with numerous cristae (arrowheads).

요 약

발아 직전에 있는 한국인삼의 접합자 배에서 유래된 자엽절편을 이용하여 체세포배를 유도하고 기내 휴면여부를 조사하고자 하였다. 식물호르몬으로써 2,4-D, BAP, kinetin을 첨가하거나 혹은 전혀 식물호르몬이 첨가하지 않은 MS배지에 절편을 배양할 경우 많은 체세포배 혹은 단일배를 획득할 수 있었다. 그러나 이런 배들은 대부분 자엽형 배(cotyledonary stage)까지 발육이 가능하지만 발아가 되지 못하여 더 이상 shoot로 생육되지 못하였고 하얀상태의 자엽형 배로 계속 유지되었다. 그러나 gibberellic acid(1.0 mg/l , GA_3)을 처리할

경우 3주 이내에 자엽형 배가 녹색으로 변하면서 발아 되었으며, 저온처리(-2 ℃에서 8주간)를 할 경우에도 체세포배가 정상적으로 발아가 되었다. 체세포배를 GA_3 및 저온 처리한 후 세포의 구조적변화를 전자현미경으로 관찰한 결과 무처리 체세포배의 자엽내 세포는 발아되지 않은 인삼접합자배의 세포와 같이 지질과립 및 세포질이 농후하며 분화되지 않은 미토콘드리아 및 엽록체를 지녀서 세포의 활성이 약한 휴면 상태의 구조를 가지고 있었다. 그러나 저온처리 및 GA_3를 처리한 자엽세포는 지질 및 세포질의 분포가 감소된 반면, 잘 발달된 엽록체와 활성이 강한 미토콘드리아의 구조를 보였다. 따라서 저온 및 GA_3 처리 후 세포의 대사활동이 활발한 것으로 보아 휴면이 타파된 것으로 판단되었다.

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