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Origin of Somatic Embryo Induced from Cotyledons of Zygotic Embryos at Various Developmental Stages of Ginseng

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Excised cotyledon segments of ginseng zygotic embryos at various developmental stages were cultured on MS basal medium from which somatic embryos were directly induced. The frequency of somatic embryo formation on the segments declined with the advancing zygotic embryo maturity. All of the cells in the cotyledons of immature zygotic embryos were smaller and more densely cytoplasmic than those in mature embryos. Histological examinations revealed that the poly-somatic embryos formed on immature embryos were of multi-cell origin and derived from the epidermal and subepidermal cell layers. However, in the cotyledon of germinating zygotic embryos, only the epidermal cells were densely cytoplasmic and singularly competent to develop into somatic embryos resulting into single embryos at a frequency of 100%.

Keywords: origin of somatic embryo, zygotic embryo, cotyledon segment, maturity of explant, *Panax ginseng*

In direct somatic embryogenesis, the embryos develop from embryogenically determined cells in the explants (Sharp *et al.*, 1980). The embryogenic cells showed a number of common features including small size, dense cytoplasm, large nuclei and small vacuoles (Thomas *et al.*, 1972). In general, the age of the cultured explant is crucial to the expression of somatic embryogenesis (Pence *et al.*, 1980; Lu and Vasil, 1982; Wang *et al.*, 1984). However, there is little information on the detailed relationship between plant age and somatic embryo development.

Somatic embryos originate from a single cell (Konar and Nataraja, 1965; Thomas *et al.*, 1972; Jones and Rost, 1989), from multiple cells (Geier and Kohlenbach, 1973; James *et al.*, 1984), or from both types on the same explants (Pence *et al.*, 1980; Williams and Maheswaran, 1986; Choi and Soh, 1993, 1994). And the somatic embryos can develop into single (Konar and Nataraja, 1965; Jones and Rost, 1989) or poly-state embryos (Williams and Maheswaran, 1986; Gui *et al.*, 1991). However, the developmental process has not been clearly elucidated.

In previous report, the ginseng cotyledon could produce somatic embryos directly on growth regulator-free medium. Most of the somatic embryos developed from near basal portions of explants without an intervening callus formation. This system is very convenient in clarifying the origin and development of somatic embryos anatomically. This experiment focused on anatomically clarifying the relationship between somatic embryogenesis and the age of the explant.

MATERIALS AND METHODS

Freshly harvested Korean ginseng (*Panax ginseng* C.A. Meyer) seeds with globular stage embryos were stratified in humidified sand for further maturation. During stratification the zygotic embryos differentiated into three states: immature cotyledon stage (1, 2 and 3 mm in size); fully mature stage (4 mm in size); and germinating stage (6 to 30 mm in size).

The seeds were immersed in 70% alcohol for 1 min and then sterilized in 1% sodium hypochlorite solution for 1 h and washed three times with distill-

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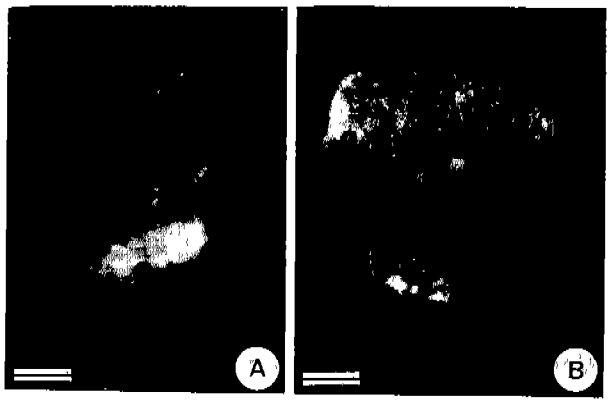

Fig. 1. Somatic embryo formation from the cotyledons of zygotic embryos at various stages. A, Somatic embryos (arrow) formed from cotyledon of a mature zygotic embryo (4 mm long) (Bar=1.7 mm); B, Somatic embryos (arrow) formed from cotyledon of a germinating zygotic embryo (6 mm long). (Bar=1.7 mm).

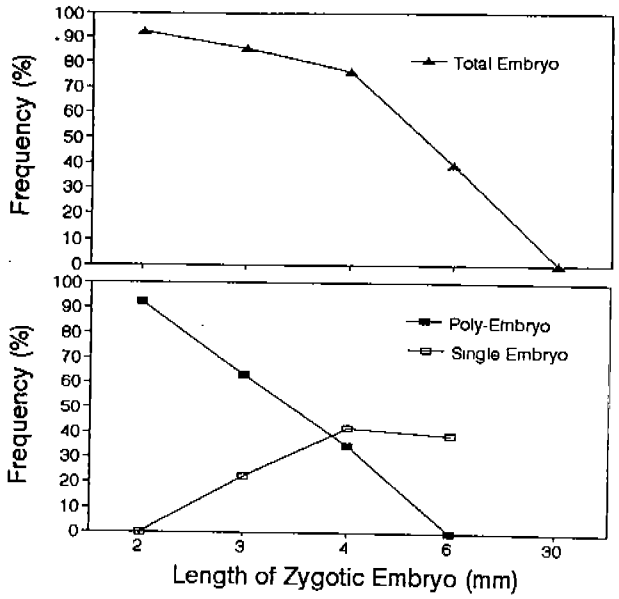

Fig. 2. Frequency of somatic embryo formation from cultured cotyledons of zygotic embryos at various developmental stages.

ed water. After carefully dissecting the zygotic embryos from the seeds, the abaxial side of excised cotyledon were placed on MS (Murashige and Skoog, 1962) medium which was composed of MS basal salts containing 3% sucrose, 0.7% agar and adjusted to pH 5.8 before autoclaving at 120℃ for 15 min. The cultures were performed using 10×1 cm glass Petridishes containing 30 mL of medium. The culture room was maintained at 24± 2℃ under 16 : 8 h photoperiods with 1900 luces by cool white fluorescent tubes. The production rate of somatic embryos was evaluated by counting cotyledon explants showing the somatic embryos from total cultured cotyledon explants. Thirty explants were cultured in each experiment which was repeated three times.

For anatomical examination, the explants were fixed in FAA (formalin, acetic acid and alcohol) and dehydrated in ethyl alcohol and then embedded in paraffin. After the samples were cut to 10 μm size, they were stained with hematoxylin. Some samples were fixed in 1% glutaraldehyde and then dehydrated with ethyl alcohol and dried in a critical point drier. After being coated with gold, the samples were observed by scanning electron microscope (JSM T 330A).

RESULTS

Direct somatic embryogenesis

The excised cotyledons of zygotic embryos were white in color just after being excised from the seed. After 4 wk culture, the cotyledons of immature and mature zygotic embryos became yellow green in color (Fig. 1A). But in the cotyledons of germinating embryos both green and red pigments accumulated (Fig. 1B).

The majority of somatic embryos developed directly from the basal portions of cotyledons without an intervening callus formation. The embryos were white in color which was comparable to green or red pigmented cotyledon tissue (Fig. 1). The highest production rate of somatic embryos (92.7± 10.23%) was observed in the cotyledon of immature zygotic embryos. Then embryogenic potential decreased as the zygotic embryos matured and germinated (Fig. 2). No somatic embryos formed in the cotyledons of seedlings grown to 30 mm long. The number of somatic embryos per cotyledon was at highest (16.7 embryos per cotyledon) in immature and mature zygotic embryos, then declined in the germinating zygotic embryos. The growth of somatic embryos was best in the cotyledons of immature zygotic embryos but then declined as the zygotic embryos grew further (Table 1).

Two types of somatic embryos were formed (Figs. 1-4). One was single embryos with a closed radicle The other was poly-embryos with lower hypocotyls fused to each other. Most cotyledon explants of the

Table 1. Number and growth of somatic embryo formed from excised cotyledons of zygotic embryos at various developmental stages

Stage	Size (mm)	No.[a] of somatic embryos per cotyledon	Maturation of somatic embryos[b]
Immature	2.0	7.5± 3.25	C
	3.0	9.7± 3.66	T, C
Mature	4.0	16.6± 9.32	G, H
Germinating	6.0	11.3± 7.24	G
Seedling	30.0	0.0	

[a]Data represents the mean values (± SD) of three replicates containing 30 explants each. [b]G, globular; H, heart; T, torpedo; C, cotyledonary embryo.

immature zygotic embryos produced poly-somatic embryos but, as the explants matured and germinated the somatic embryos developed into a singular state.

Origin of somatic embryos and explant age

The somatic embryogenesis of cotyledon explants of ginseng was observed both morphologically and anatomically. The cells in the cotyledons of immature zygotic embryos (3 mm long) were small and densely cytoplasmic before culture (Fig. 4A). After 5 d culture small nodules formed from an irregular division of multiple cells in the epidermis and subepidermis (Figs. 3A, 4B). After 5 wk culture the nodules developed into poly-somatic embryos with lower hypocotyls fused to each other (Figs. 3B, 4C). Before culture in the cotyledons of mature zygotic embryos (4 mm in size), only epidermal cells were small and densely cytoplasmic but the subepidermal cells were large and vacuolated (Fig. 4D). Periclinal division occurred from epidermal cells which gave rise to a number of globular nodules after 5 d of culture (Figs. 3C, 4E) but no division was observed from the subepidermal cells. Poly and single embryos (Figs. 3D, 4F) formed in mature zygotic embryos. In germinating zygotic embryos, most cells of the cotyledons were vacuolated except for certain single epidermal cells which were still small and dense cytoplasm (Fig. 4G). After 5 d culture, these single cells divided periclinally and anticlinally and formed early globular somatic embryos (Figs. 3E, 4H). Subsequently, single somatic embryos with closed radicles were formed (Figs. 3F, 4I).

DISCUSSION

The highest production rate of somatic embryos was observed in the excised cotyledon explants of immature zygotic embryos. Concurrently, embryogenic potential decreased as the zygotic embryos matured and germinated. This result corresponded with the tissue culture of *Zea mays* zygotic embryos in which the immature zygotic embryos showed high embryogenic competency but a reduced embryogenic capacity of mature zygotic embryos due to loss of embryogenic potential (Green and Phillips, 1975). In general, embryogenic potential was highly influenced by the age of the explants (Pence *et al.*, 1980; Ghazi *et al*, 1986; Gingas and Lineberger, 1989).

Generally, the culture medium contains growth regulators which do not induce preferential growth of embryogenic cells since nonembryogenic cell division is also affected by growth regulators (Pence *et al.*, 1980; Williams and Maheswaran, 1986). In our system, the medium did not contain any exogenous growth regulators, therefore only embryogenic division was stimulated. This result indicates all cell division in our cultured cotyledon tissue was in embryogenic cell. From this observation the distribution of embryogenic cells came to be highly different from the explant age. The embryogenic cells were distributed in all areas of the cotyledons of the immature zygotic embryos. After the zygotic embryos matured the embryogenic cells were restricted to the epidermis. When the zygotic embryos germinated, small and densely cytoplasmic cells were remained singly among vacuolated epidermal cells. This means that the embryogenic capacity of cotyledons of zygotic embryos correlated with the residue of undifferentiated meristematic cells since embryonic development occurred from small and densely cytoplasmic cells and the high embryogenic potential was associated with a large number of embryonic cells in the cotyledon explants.

Somatic embryos originated from single cells (Konar and Nataraja, 1965; Thomas and Street, 1972; Jones and Rost, 1989), from multiple cells (Geier and Kohlenbach, 1973; James *et al.*, 1984) or from both ways on the same explants (Pence *et al.*, 1980; Williams and Maheswaran, 1986; Choi and Soh, 1993, 1994). In the present experiment, somatic embryos originated from multiple cells when the embryogenic cells were in a massive state in an imma-

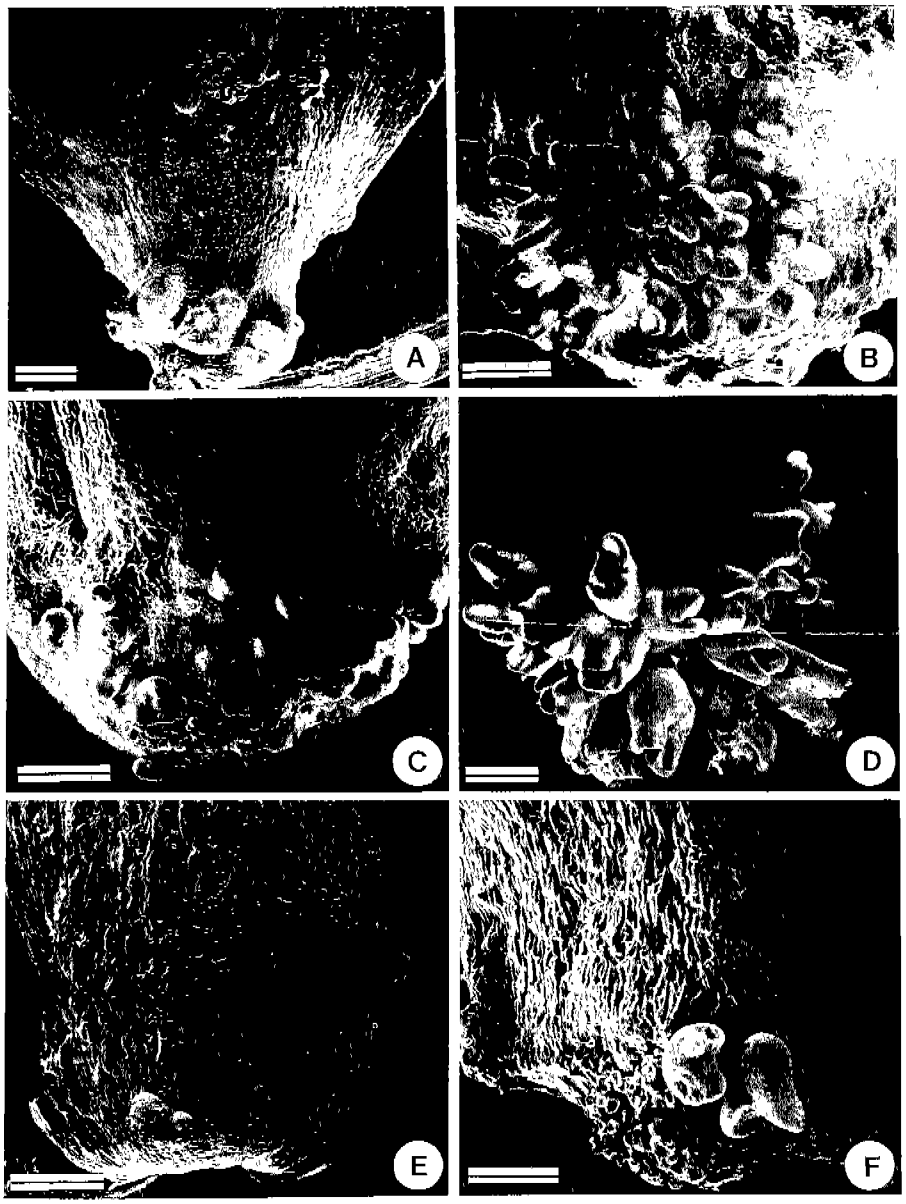

Fig. 3. Scanning electron microscopy of somatic embryo development from cotyledons of zygotic embryos at various developmental stages. A-B, Poly-somatic embryos (arrow) formed from cotyledon of an immature zygotic embryo (3 mm long) (Bar=500 μm); C-D, Poly- and single embryos formed from cotyledon of a mature zygotic embryo (4 mm long) (Bar=500 μm); E-F, Single embryos formed from cotyledon of a germinating zygotic embryos (6 mm long) (Bar=500 μm).

ture zygotic embryo. In germinating zygotic embryos, somatic embryos originated from single cells which were located singly among the vacuolated cells of epidermis. This could mean that the cellular origin of somatic embryos simply may dependent on whether the embryogenic cells were located in a single or aggregated states in the tissue.

The present experiment showed two types of embryo development such as single embryos with a closed radicle and poly-somatic embryos which fused to each other. There is some evidence that undifferentiated tissue such as immature zygotic embryos of *Ilex, Trifolium, Acanthopanax* produced poly-embryos (Hu and Sussex, 1972; Maheswaran and Williams, 1985; Gue *et al.*, 1991) and differentiated tissue such as seedling *Ranunculus* produced single embryos (Konar and Nataraja, 1965). The poly somatic embryos generally arose from multiple cells but not from

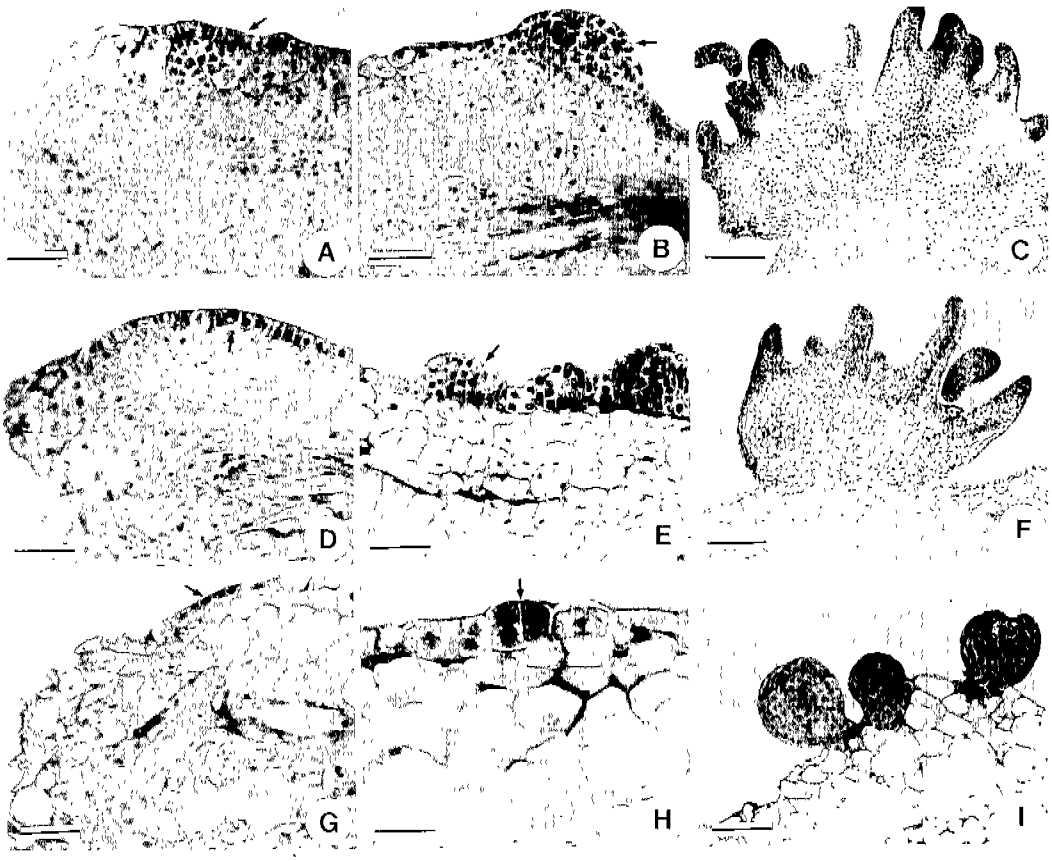

Fig. 4. Histological observation of somatic embryo development from cotyledons of zygotic embryos at various developmental stages. A-C, Somatic embryogenesis from cotyledons of immature zygotic embryos; A cotyledon constituted of small cells and with dense cytoplasm in an immature zygotic embryo (Bar=82 μm); B, Initiation of somatic embryogenesis (arrow) from epidermal and subepidermal multiple cells (Bar=82 μm); C, Formation of poly-somatic embryos from the cotyledon of an immature zygotic embryo (Bar=330 μm); D-F, Somatic embryogenesis from cotyledons of mature zygotic embryos; D, Only the epidermal cells were embryogenic (arrow) in cotyledon of mature zygotic embryo (Bar=82 μm); E, Initiation of somatic embryogenesis (arrow) from multiple epidermal cells (Bar=82 μm); F, Formation of poly-embryos from the epidermis of cotyledon (Bar=330 μm); G-I, Somatic embryogenesis from cotyledons of germinating zygotic embryos; G, Some epidermal single cells being embryogenic (arrow) in the cotyledon of a germinating embryo (Bar=82 μm); H, Initiation of somatic embryogenesis from epidermal single cells (arrow) (Bar=23 μm); I, Formation of single globular embryos (Bar=82 μm).

single cells. Based on the above results, we suspect that the poly-embryos originated from multiple cells might have been formed from the multiple initiation of somatic embryos on the same portion. It is assumed that the factors influencing the origin of somatic embryos correspond to the coordinated behaviour of cells participating in embryonic development (Williams and Maheswaran, 1986).

In the present study, somatic embryogenesis occurred from cultured cotyledons on medium without supplemented growth regulators. Little work has been done in somatic embryogenesis on culture media without growth regulator addition (Hu and Sussex, 1972; Smith and Krikorian, 1989). Stimulation of embryonic development from explants was not clearly resolved except osmotic stress (Kamada *et al.*, 1989) and physical wounding related to somatic embryo formation (Smith and Krikorian, 1989). The factors stimulating somatic embryo development from cultured cotyledons of ginseng zygotic embryos on growth regulator-free medium are under examination.

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成熟度가 다른 人蔘 接合子胚의 子葉으로부터 形成되는 體細胞胚의 起源

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적 요

성숙도가 다른 접합자배의 자엽을 MS 기본배지에 배양하여 체세포배 발생기원을 관찰하였다. 체세포배의 발생률은 미숙배의 자엽에서 가장 높았고 성숙될수록 감소되어 발아된 유식물에서는 체세포배가 발생되지 않았다. 미숙배의 자엽을 구성하고 있는 세포는 성숙배의 자엽세포보다 크기가 작고 세포질이 농후하였다. 이와 같은 경우는 체세포배가 다세포로부터 기원되어 다배로 발생되었다. 한편 발아중에 있는 접합자배의 자엽은 표피에 부분적으로 한정된 세포만이 크기가 작고 세포질이 농후한 세포의 분포가 관찰되었다. 이와 같은 경우에 체세포배는 표피의 단세포로부터 기원되어 단독배로 발생되었다. 따라서 체세포배의 기원이나 발생양상은 배양조직의 성숙과정에서 세포분화에 따라 현저히 달라짐을 알 수 있었다.

주요어: 체세포배의 기원, 접합자배, 자엽절편, 배양재료의 성숙도, 인삼

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