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Developmental and structural aspects of somatic embryos formed on medium containing 2,3,5-triiodobenzoic acid

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Abstract Cotyledon explants of ginseng (*Panax ginseng* C.A. Meyer) zygotic embryos produced somatic embryos at a high rate (68%) on medium without any growth regulators. Under this culture condition, apparent polar somatic embryogenesis occurred near the basal-excised portion of the cotyledons. When the cotyledon explants were cultured on medium containing 2,3,5-triiodobenzoic acid (TIBA), an auxin polar-transport inhibitor, the frequency of somatic embryo formation markedly decreased and was completely inhibited on medium containing 20 μ M TIBA. On medium containing 5–10 μ M, somatic embryos developed sporadically on the surface of the cotyledons and had a normal embryo axis but jar-shaped cotyledons. Embryos with jar-shaped cotyledons were also observed to occur at a high frequency when the early globular embryos formed on hormone-free medium were transferred to medium containing 20 μ M TIBA. From these results, it was deduced that endogenous auxin in the cotyledon explants plays an important role in the induction of somatic embryos and that the cotyledon development in somatic embryos is also related to the polar transport of endogenous auxin.

Key words Somatic embryogenesis · Cotyledon morphology · *Panax ginseng* · Auxin transport inhibitor

Abbreviations MS Murashige and Skoog (1962) · TIBA 2,3,5-triiodobenzoic acid

Introduction

Somatic embryogenesis in plant tissue cultures has been considered a model system for the understanding of plant embryo development, and exogenously supplied growth regulators are essential to the process of somatic embryogenesis. Different concentrations and different types of exogenous growth regulators are required by each plant species (Ammirato 1983). However, once somatic embryo development commences, the further process of somatic embryogenesis continues on medium without growth regulators, and this observation is supported by the fact that exogenous growth regulators must be omitted for normal somatic embryo development in many plant tissue cultures (Ammirato 1983). In this context, it is suspected that the morphologic development of somatic embryos is regulated by endogenous hormones. However, the role of endogenous hormones has been neglected by the masking effects of exogenously supplied growth regulators, and little progress has been achieved toward understanding the role of endogenous plant hormones in plant embryo development.

In some plants, somatic embryos can be formed from cultured explants on medium without any growth regulators (Hu and Sussex 1971; Smith and Krikorian 1989; Kamada et al. 1989; Choi and Soh 1994). This means that the entire process of somatic embryogenesis is normally regulated by only endogenous hormones. In *Panax ginseng*, we observed that the excised cotyledon segments directly produce somatic embryos on medium without any growth regulators (Choi and Soh 1994, 1996a,b). In this case of somatic embryogenesis endogenous factors of explants influenced the initiation and development of embryos: the embryo axis contained a substance suppressing somatic embryo development, thus detachment of the cotyledon from the embryo axis was required for somatic embryogenesis (Choi and Soh 1996b). Furthermore, polar somatic embryogenesis from cotyledon segments was induced by the cooperative actions of both wound response and tissue polarity of the explants (Choi and Soh 1996a). From this

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process it can be suggested that endogenous hormones have a role in somatic embryogenesis.

Polar auxin transport has long been postulated to play a central role in plant embryogenesis, and the disturbance of auxin polar transport by TIBA results in the anomalous cotyledon morphology of zygotic embryos in *Brassica juncea* (Liu et al. 1993). Since somatic embryos in an *in vitro* culture system show a great diversity in structure (Soh 1993), we examined the effects of a polar auxin transport inhibitor, TIBA, on developmental and structural aspects of somatic embryos originating from the culture of ginseng cotyledons.

Materials and methods

The seeds of Korean ginseng (*Panax ginseng* C.A. Meyer) were harvested from the field of the Korean Ginseng and Tobacco Research Institute. The seeds had extremely immature embryos just after harvest, but the embryos in the seeds grew fully to 6 mm in length when the seeds were stored by stratification at a low temperature for several months. In preparation for treatment, the seeds were immersed in 70% alcohol for 1 min, sterilized in 1% sodium hypochlorite solution for 1 h, and then rinsed three times with aseptic distilled water. Excised cotyledon segments were placed with their adaxial side on MS (Murashige and Skoog 1962) basal salts containing 3% sucrose and 0.7% agar in 10×1-cm plastic petri dishes containing 30 ml of medium. To observe the effect of TIBA on the formation of somatic embryos, we added varied concentrations of TIBA to the medium. The medium was adjusted to pH 5.8 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. The rate of production of somatic embryos was evaluated by counting cotyledon explants producing somatic embryos from the total number of cultured cotyledon explants. Thirty explants were cultured in each experiment. Each experiment was performed three times.

The effect of TIBA on the development of somatic embryos was observed by transferring those cotyledon explants having globular somatic embryos at an early stage (30–50 μm in length) and at a late stage (100–150 μm in length) which were formed on TIBA-free medium to MS basal medium without TIBA and MS basal medium containing varied concentrations of TIBA in 10×1-cm plastic petri dishes containing 30 ml of medium. Fourteen cotyledon explants having globular embryos were cultured in each petri dish. Five replicates were used per treatment. Each experiment was performed three times.

Some samples were fixed in 1% glutaraldehyde and dehydrated with ethyl alcohol and then dried in a critical point drier. After being coated with gold, the samples were observed by a scanning electron microscope (JSM T330A).

Results

Effect of TIBA on somatic embryogenesis

In a previous study, it was found that somatic embryos originated from single cells of the epidermis of ginseng cotyledons (Choi and Soh 1994). In the present investigation, on TIBA-free medium polar somatic embryogenesis occurred directly near the basal-excised portion of the cotyledons without any intermediate callus formation

(Fig. 1A, B) and 68% of the explants produced somatic embryos. Somatic embryos developed into the globular stage after 3 weeks of culture (Fig. 1A), then successively into the heart-shaped (Fig. 1B), torpedo (Fig. 1C) and cotyledonary stages (Fig. 3D).

Cotyledon explants of ginseng were cultured on MS basal medium containing concentrations of TIBA varying from 5 to 20 μM. In such cultures, the frequency of somatic embryo formation from cotyledon explants was highly suppressed parallel to increasing concentrations of TIBA (Fig. 2). Cotyledon explants did not produce somatic embryos on medium containing 20 μM TIBA and deteriorated to a necrotic brown color. Somatic embryos formed on medium containing 5–10 μM TIBA developed sporadically on the whole surface of the cotyledons instead of undergoing polar development (Fig. 1D). All of the embryos had tubular or jar-shaped cotyledon structures (Fig. 1D, arrows) which developed without the intervening heart-shaped stage showing bilateral cotyledons. However, embryo axes developed normally.

Effect of TIBA on the cotyledon morphology of embryos

Early globular somatic embryos at about 30–50 μm in length which formed on hormone-free medium were transferred onto medium containing TIBA at concentrations varying from 5 to 20 μM. In TIBA-free medium, light and scanning electron microscopy revealed that the globular somatic embryos (Fig. 3A) developed into the heart-shaped embryos with separate cotyledonary primordia (Fig. 3B), which subsequently matured with well-developed cotyledons (Fig. 3C, D). Of the somatic embryos grown on hormone-free medium 46% had two cotyledons and 54% had more than two (poly) (Figs. 3D, 4). On TIBA-containing medium, cotyledon primordia were formed circularly on the tops of globular embryos (Fig. 1E), from which tubular cotyledons developed (Figs. 1F, 5A), and then embryos with jar-shaped cotyledons formed as they matured (Figs. 1G, 5B). The shoot apex region of somatic embryos with jar-shaped cotyledons was very small (Fig. 5D), but normal somatic embryos had a broad shoot apex (Fig. 3B). The axial growth (root and shoot poles) of embryos was not influenced by TIBA treatment (Figs. 1G, 5B). The frequency of jar-shaped cotyledon formation increased markedly as the concentration of TIBA increased up to 20 μM (Fig. 4), but in concentrations over 40 μM TIBA, globular embryos slowly deteriorated and browned. When the somatic embryos at the proembryonic stage constituting about 8–30 cells (under 20 μm in size) were cultured on medium containing 20 μM TIBA, they slowly deteriorated without further development. In the culture of late-globular embryos over 100 μm in length on medium with 20 μM TIBA, the embryos had a furrow (Fig. 5C, arrow) on the margin cotyledon of jar-shaped, but slightly jar-shaped cotyledons (Fig. 5C) were frequently observed.

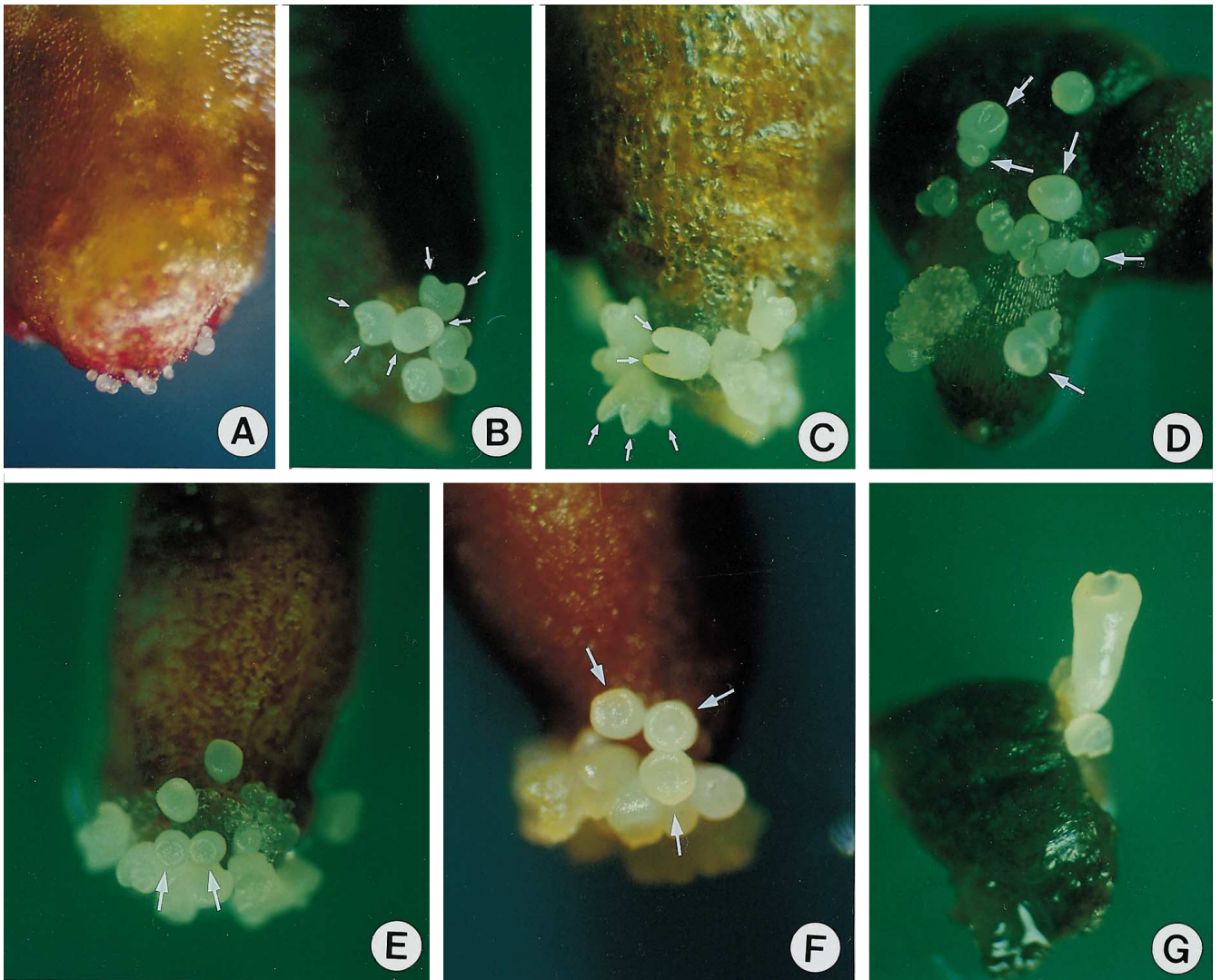


Fig. 1A–G Somatic embryos formed from ginseng cotyledons on medium with and without TIBA. **A** Polar development of globular somatic embryos on near the basal-excised portion of ginseng cotyledons on hormone-free medium after 3 weeks of culture. **B** Heart-shaped embryos with two cotyledon primordia (*arrows*) on hormone-free medium after 4 weeks of culture. **C** Torpedo embryos with discrete cotyledons (*arrows*) on hormone-free medium. **D** Somatic embryos with jar-shaped cotyledons (*arrows*) formed sporadically from a ginseng cotyledon on medium containing 5 μM TIBA. **E** Embryos with circular ring-like cotyledon primordia (*arrows*) after transfer of early-globular embryos to medium containing 20 μM TIBA. **F** Embryos with jar-shaped cotyledons (*arrows*) on medium containing 20 μM TIBA. **G** Mature embryos with jar-shaped cotyledons on medium containing 20 μM TIBA

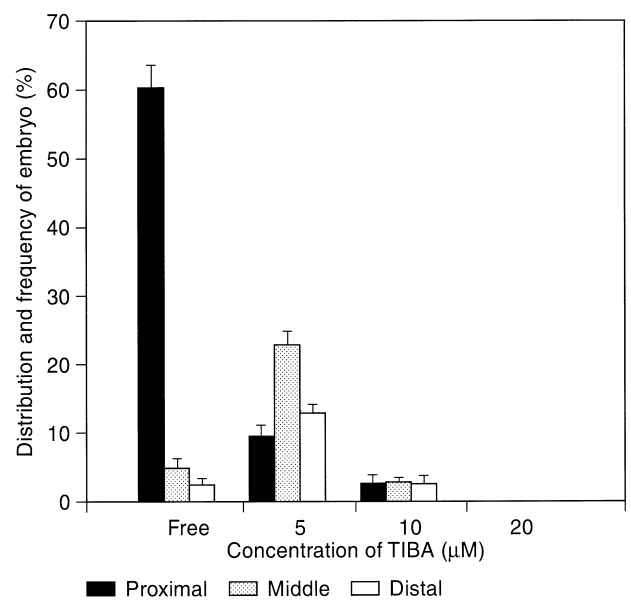


Fig. 2 Effects of TIBA on the frequency of embryo formation and the distributional patterns of embryos during the culture of ginseng cotyledons. Data represent the mean values ±SD from three independent experiments

Fig. 3A–D Scanning electron micrography of somatic embryos after the transfer of early-globular embryos to TIBA-free medium. **A** Globular somatic embryos after 1 week of culture (bar: 153 μm). **B** Heart-shaped embryos with discrete cotyledons (arrows) after 2 weeks of culture (bar: 153 μm). **C** Torpedo-shaped embryos (arrows) after 3 weeks of culture (bar: 238 μm). **D** Fully mature embryos with well-developed cotyledons after 5 weeks of culture (bar: 400 μm)

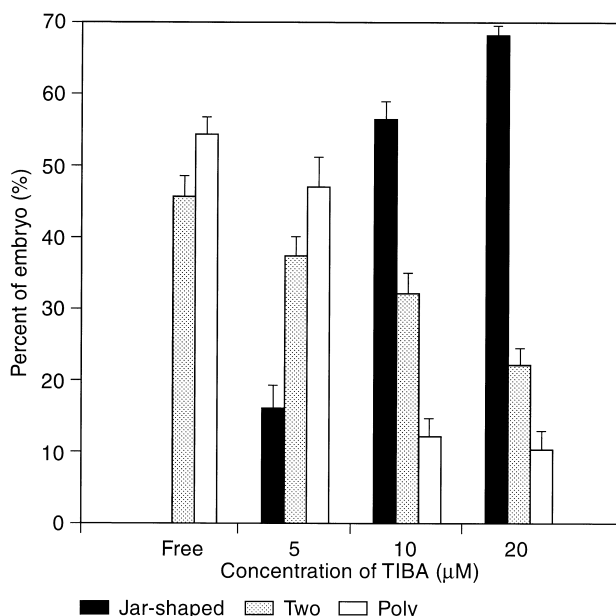
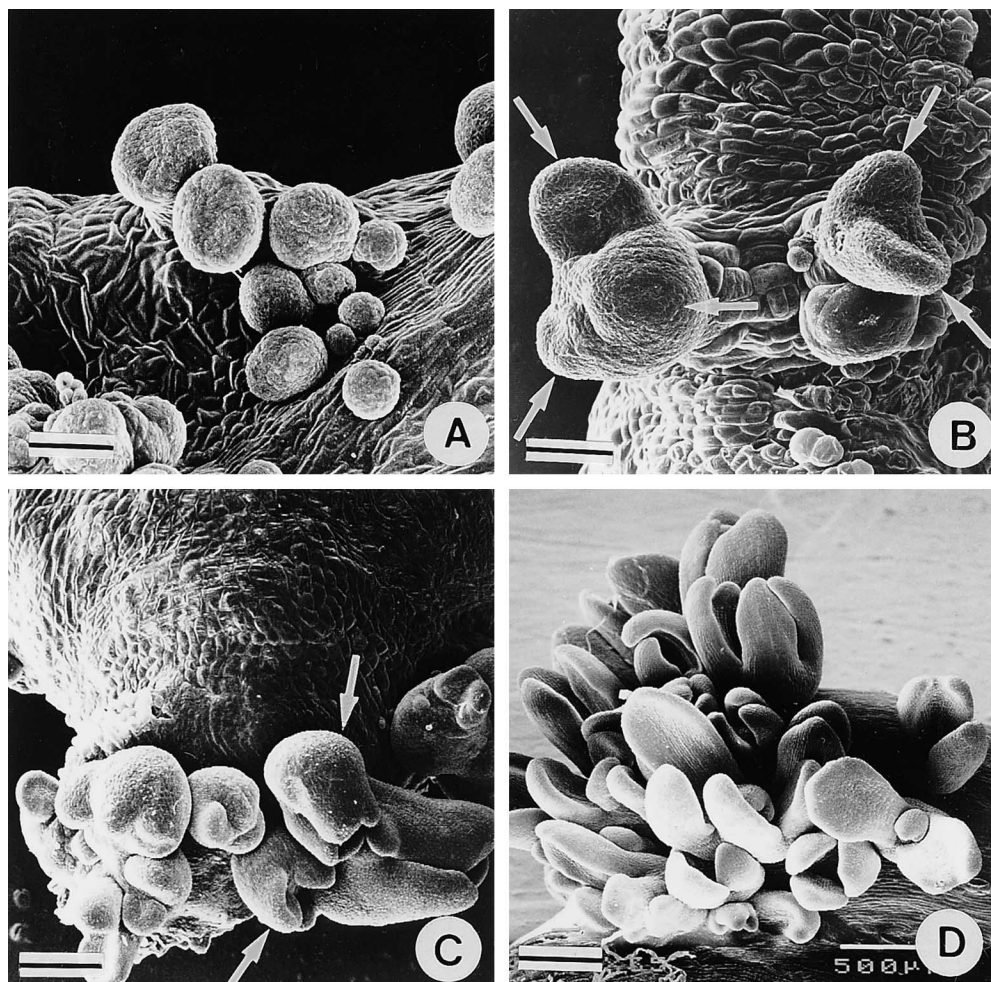


Fig. 4 Effects of TIBA on the increase of jar-shaped cotyledons of somatic embryos after the transfer of early-globular somatic embryos to MS basal medium with varied concentrations of TIBA. Data represent the mean values \pm SD from three independent experiments

Discussion

Polar somatic embryogenesis occurs near the basal-excised portion of ginseng cotyledon segments when cultured on hormone-free medium. In a previous experiment, somatic embryogenesis from ginseng cotyledon segments was induced by the cooperative action of both wound response and the tissue polarity of explants (Choi and Soh 1996a). It was known that the polar development of organs is closely related to the polar accumulation of endogenous auxin (Moncousin et al. 1989). Therefore, the factor inducing somatic embryos from ginseng cotyledons was thought to be related to the polar accumulation of endogenous auxin in the basal portion of the cotyledons. Based on the results of the previous experiments, we decided to investigate the role of endogenous auxin on ginseng somatic embryogenesis by TIBA treatment. On medium with TIBA, somatic embryos developed sporadically on the surface of the cotyledon segments, and the TIBA treatment strongly suppressed the somatic embryo production. The results indicated that the polar accumulation of endogenous auxin to the basal portion of the cotyledons induced somatic embryos from that area, while any interference of auxin po-

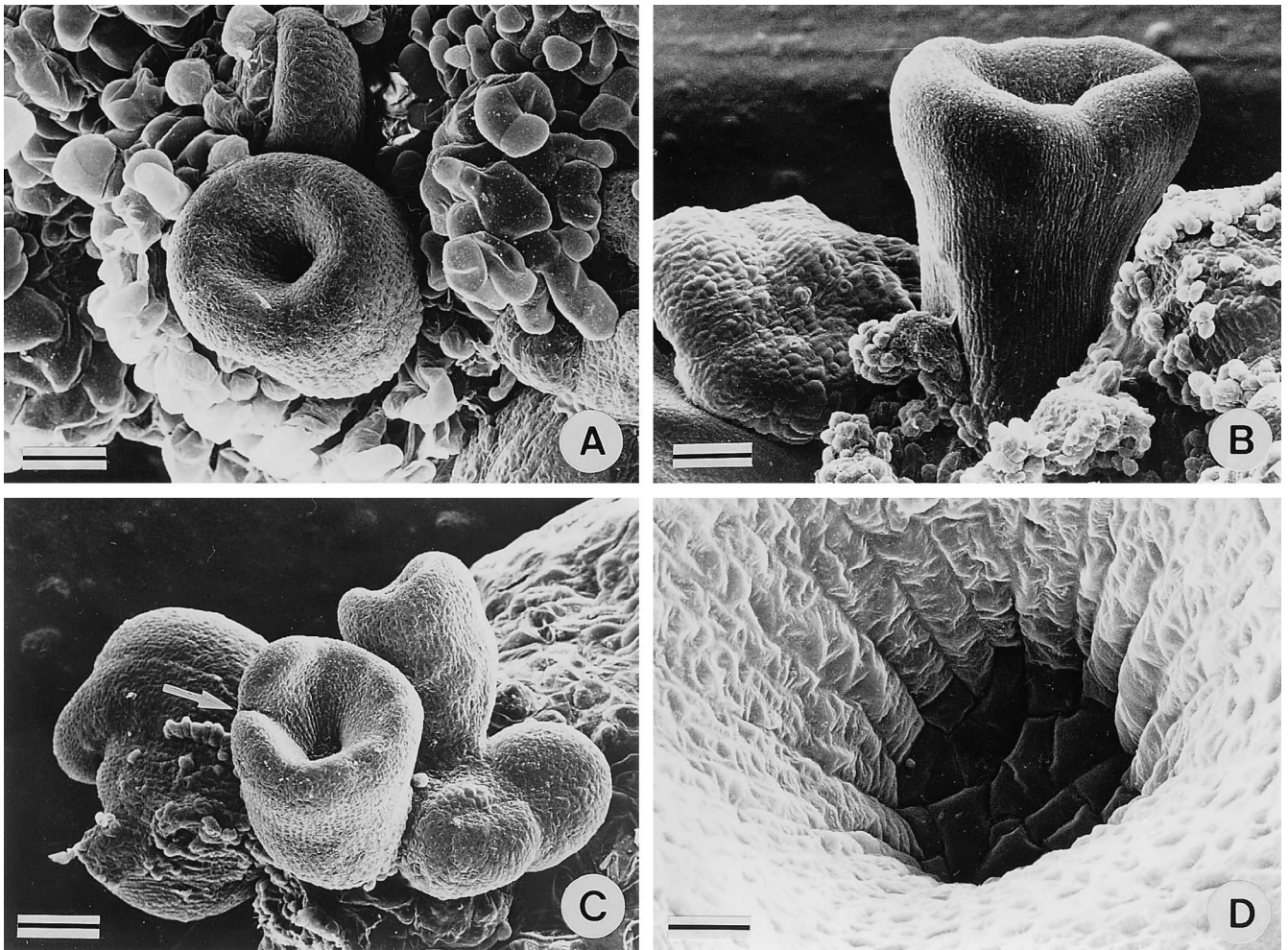


Fig. 5A–D Scanning electron micrography of somatic embryos after the transfer of early-globular embryos to medium containing $20 \mu\text{M}$ TIBA. **A** Somatic embryo with tubular or jar-shaped cotyledons on TIBA-containing medium after 2 weeks of culture (*bar*: $71 \mu\text{m}$). **B** Somatic embryo with jar-shaped cotyledons after 4 weeks of culture on TIBA-containing medium (*bar*: $238 \mu\text{m}$). **C** Embryos with jar-shaped cotyledons with a furrow on the cotyledon margin (*bar*: $149 \mu\text{m}$). **D** Magnified view of nearly apical regions of a embryo with jar-shaped cotyledon (*bar*: $13 \mu\text{m}$)

lar transport by TIBA treatment resulted in the scattered development of somatic embryos on the cotyledon surface.

The possible role of polar auxin transport on the morphologic development of somatic embryos was tested by Schiavone and Cooke (1987), who treated globular stages of carrot somatic embryos with TIBA. Globular embryos underwent persistent spherical expansion without successive development from the heart-shaped to the torpedo stages. In contrast to Schiavone and Cooke's observations, Liu et al. (1993) reported that similarly treated globular zygotic embryos of *Brassica juncea* exhibited typical axial elongation and that the cotyledons emerged as jar-shaped structures. Cooke et al. (1993) suggested that the

different morphological effects of TIBA on the embryo development between *B. juncea* and carrot embryos might be caused by an overlapping effect of a positional mechanism that arises as a maternal effect from the ovular tissue surrounding the zygotic embryo and polarized position of the egg and/or early embryo within the embryo sac. In their opinion, carrot somatic embryos lacked the positional mechanism because they developed from embryogenic cell clumps in a liquid medium; therefore, the axial polarity and cotyledon development of carrot somatic embryos are profoundly affected by the polar auxin transport inhibitor. In the present experiment, we observed the effects of TIBA on the development and structure of ginseng somatic embryos after transfer of the globular embryos to medium containing TIBA. In this culture, the morphologic development of ginseng somatic embryos in the presence of TIBA was rather similar to that of zygotic embryos of *Brassica juncea* (Liu et al. 1993) because the developed ginseng somatic embryos had a normal embryo axis with jar-shaped cotyledons. The ginseng somatic embryos were not cultured under the same conditions as the carrot somatic embryos of Schiavone and Cooke (1987) because the carrot somatic embryos develop in a freely suspended state in liquid medium, while ginseng somatic embryos always

develop directly and perpendicularly on the surface of cotyledon explants.

Plant embryos have clear axial polarity, with shoot and root poles. Auxin transport assays have shown that polar auxin transport in mature embryos occurs toward the root end (Fry and Wangermann 1976). It is suspected that if the somatic embryos were initiated on medium in the presence of TIBA, the axial polarity of somatic embryos may be influenced. To verify this suspicion, we observed the morphology of somatic embryos which were initiated and developed in the presence of TIBA: the axial polarity of the somatic embryos were not affected. This result means that the attainment of axial polarity of somatic embryos might not be controlled solely by auxin polar transport inhibitors. Generally in somatic embryogenesis, in contrast to zygotic embryogenesis, embryo development begins with the formation of an irregularly segmented cell complex without obvious axial polarity (Halperin 1964). Haccius and Bhandari (1975) concluded that the establishment of polarity in somatic embryos is delayed because of their indefinite positions in relation to their environment. But it is still not known precisely what mechanism is involved in attaining polarity in somatic embryos.

It was known in rape seed that the attainment of bilateral symmetry from axial symmetry in plant embryogeny occurs at the time of transition from the globular to the heart-shaped stage (Tykarska 1979). In the culture of ginseng embryos at the late-globular stage on medium containing 20 μ M TIBA, somatic embryos had jar-shaped cotyledons but with a furrow on the cotyledon margin. This type of cotyledon seems to be an intermediate type between jar-shaped and large, like those of carrot somatic embryos (Soh et al. 1996). This indicates that bilateral symmetry of somatic embryos is already determined in the late-globular stage. Therefore, in somatic embryos at a more advanced stage than the late globular stage, bilateral symmetry determined by polar auxin transport may not be fully disturbed by auxin transport inhibitor.

Generally, zygotic embryos of dicotyledonous plants always have two discrete cotyledons lateral to the embryo axis, but in somatic embryos the cotyledon number shows great diversity (Soh 1996). In the present experiment, a large number of ginseng somatic embryos had multiple cotyledons, even though they developed on hormone-free medium. It is not clear how this diversity of cotyledon number arises in somatic embryos. However, there is some evidence of the factors involved in diversifying cotyledon number. Continuous ABA treatment increases somatic embryo formation with anomalous cotyledons (Lee and Soh 1994). Cytokinin treatment also increases the number of somatic embryos with multiple cotyledons (Ammirato 1977; Lee and Soh 1993b). From these results, we surmise that unbalanced endogenous hormone distribution by exogenous hormone treatment may result in the abnormalities of somatic embryos. Somatic embryos with bowling pin and jar-shaped cotyledons are frequently observed when the somatic embryos are formed on medium containing high concentrations of 2,4-D (Lee and Soh 1993a). This is supported by carrot cell culture in which 2,4-D treat-

ment actually stimulates the accumulation of large amounts of endogenous IAA (Michalczuk et al. 1992), and by soybean tissue culture in which the production of somatic embryos with jar-shaped cotyledons occurs on medium containing 2,4-D at relatively high levels (Choi et al. 1994).

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