*Journal of Plant Biology*, September 2000, 43(3) : 115-120

# Relationship between Auxin-Induced Cell Proliferation and Somatic Embryogenesis in Culture of Carrot Cotyledons

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**We elucidated the relationship between cell proliferation and somatic embryogenesis in the culture of carrot cotyledons. Fresh weights of the cotyledon explants were determined every five days while being cultured on a medium containing 2,4-D. Callus production increased exponentially from Day 20 to Day 25, showing a two-fold rate of proliferation. To examine the embryogenic potential of the callus, we pre-cultured cotyledon explants on an MS medium with 2,4-D, then transferred them to an MS basal medium at five-day intervals. Somatic embryos formed most frequently when the cotyledons were pre-cultured for 20 days on an MS medium that contained 5 μM 2,4-D. The frequency of somatic embryo formation was 81%, while that of normal embryos with two cotyledons was 51% among those formed on a hormone-free medium. We used FACScan analysis to relate the embryogenic potential of the callus to the S phase in the cell cycle of cultured cells. The S phase was high after 25 days of culture on the medium with 5 μM 2,4-D. In contrast, the frequency of normal embryogenesis was higher at Day 20 of the pre-culture period. Culturing embryogenic calli on a medium with 5 μM 2,4-D was most favorable for producing somatic embryos with two cotyledons. We verified that active somatic embryogenesis was apparently related to cell division activity; somatic embryos induced from actively dividing cells were apt to accompany cotyledonary abnormality.**

*Keywords*: anamalous somatic embryogenesis, cell division, 2,4-D, *Daucus carota* L.

Dedifferentiation of plant cells in culture systems is an important process for somatic embryogenesis and organogenesis. This dedifferentiation is a transitional phenomenon in cell cycles, from the $G_0$ to the S phase, and refers to a loss of differentiation. The plant growth regulator 2,4-D stimulates not only the dedifferentiation of differentiated tissues but also embryogenic potential (Komamine, 1991). In addition, 2,4-D induces active proliferation of dedifferentiated cells, whereas somatic embryos develop on media that lack 2,4-D. Therefore, somatic embryogenesis is related to the frequency of cell division as well as the induction and proliferation of dedifferentiated cells.

Mass production of normal somatic embryos, is required for the development of plant biotechnology methods, as well as for scientific purposes. However, some problems remain in the production of somatic embryos, such as the appearance of abnormal embryos and low germinability (Lazzeri et al., 1985; Soh, 1993; Choi et al., 1994; Soh et al., 1997). Adding 2,4-D to the culture medium promotes the development of abnormal cotyledons. For example, cotyledon development in soybean embryos was arrested when they were produced on a medium containing a high concentration of 2,4-D (Choi et al., 1994). Likewise, the structural abnormality of somatic embryos depended on the concentration and period of 2,4-D treatment in the medium (Konan et al., 1994). Cotyledon abnormality in *Aralia cordata* was induced during long-term culture on a medium with 2,4-D (Lee and Soh, 1993a). Therefore, because the production of normal somatic embryos with two cotyledons is so essential, we attempted to verify the relationship between cell proliferation of cultured tissues and somatic embryogenesis, and to clarify the effects of both concentration and treatment period of 2,4-D on the development and structure of somatic embryos.

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## MATERIALS AND METHODS

### Plant Materials and Culture Condition

Carrot seeds (*Daucus carota* L. cv. Hongshim) were sterilized first with 70% ethanol for 1 min, then with a 1% sodium hypochlorite solution for 10 min. They

were then rinsed four times with sterile distilled water. The seeds were cultured in a 100-mL Erlenmeyer flask containing 1/2 MS (Murashige and Skoog, 1962) medium that was adjusted to pH 5.8 before autoclaving. The cotyledon explants (3 × 3 mm) of seven-day-old seedlings were cultured in Petri dishes (60 × 15 mm) containing 10 mL MS medium. This medium had been supplemented with 5 to 50 μM 2,4-D (Sigma, USA), 30 g/L sucrose, and 8 g/L agar (Lee et al., 1997). All cultures were maintained at 25 ± 1°C and a 16 h photoperiod, with cool white fluorescent lights of 24 μmol $m^{-2}$ $s^{-1}$. At five-day intervals, we determined the fresh weight of each explant that had been cultured on the medium containing 5 μM 2,4-D.

### Analysis of Cell Cycles

The analysis of cell cycles was determined with a modified method reported by Bharathan et al. (1994). Explants cultured on the medium with 5 μM 2,4-D were fixed with 70% ethanol every five days. The explants were frozen with liquid nitrogen in a vessel. Samples were mixed with 5 mL of chopped buffer (pH 7.0) that comprised of 45 mM $MgCl_2$, 30 mM sodium citrate, 20 mM MOPS, and Triton X-100 (0.05% w/v). They were then filtered through a 20-μm nylon mesh. The nuclear suspension was treated with ribonuclease (100 μL, 1 mg/mL) and stained with propidium iodide (1 mL, 50 μg/ mL). After incubating 30 min in the staining solution, the samples were analyzed with a FACScan (Becton Dickinson, Mountain View, CA., USA). Total DNA contents were determined after excitation of the samples with a 5-mW laser at 488-nm, and red fluorescent light was collected with a 585-nm filter. Data were analyzed with the software program LYSIS II (Becton Dickinson).

### Somatic Embryogenesis from Cultured Cells

Cotyledon explants from seven-day-old seedlings were cultured for 20 days on an MS medium containing 1 to 50 μM 2,4-D. They were then transferred to a 2,4-D-free medium, where they were cultured an additional three weeks for somatic embryogenesis. The frequency of somatic embryogenesis was determined 21 days after culturing on the 2,4-D-free medium in the dark. Five weeks into this culture period, we examined the somatic embryos for the shape and number of their cotyledons.

## RESULTS

Explant fresh weights increased prominently from Day 20 to Day 30 after they were placed on the culture media. Fresh weights then remained almost constant until Day 35 (Fig. 1). This increment during callus growth and somatic embryogenesis followed a sigmoidal curve. As shown on the DNA histogram of cycles in the explant cells (Fig. 2), the 4C contents increased slightly within 10 days of culturing. Changes in DNA contents from 2C to 4C were observed within 25 days post-culture; 4C contents decreased after 30 days. The S phase, from Day 5 to Day 25 increased 7.7-fold (Table 1), then decreased 30 days after cul-

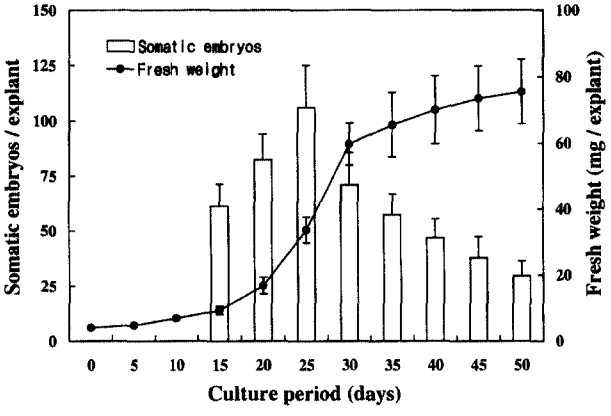


**Figure 1.** Effect of culture period in media containing 2,4-D on the proliferation of cells (fresh weight) and somatic embryogenesis in carrot explant cultures. Explants were cultured for various periods on MS solid media with 5 μM 2,4-D, then transferred onto MS solid media without 2,4-D for embryogenesis for 20 days. Fresh weight of cultured explants was measured at five-day intervals. The bars represent mean ± SE (n = 5).

**Table 1.** Changes in cell-cycle phases over the culture period for carrot cotyledon explants on a medium containing 5 μM 2,4-D.

| Culture (days) | Cell number (%) | | | |
|---|---|---|---|---|
| | $G_1$ | S | $G_2$/M | |
| 5 | 95.9 ± 4.1 | 1.8 ± 0.3 | 1.5 ± 0.2 | 0.02[a] |
| 10 | 89.0 ± 4.4 | 2.1 ± 0.5 | 4.6 ± 0.4 | 0.02 |
| 15 | 78.7 ± 3.1 | 11.6 ± 0.6 | 7.4 ± 0.3 | 0.15 |
| 20 | 66.3 ± 3.0 | 12.7 ± 0.4 | 15.5 ± 0.8 | 0.19 |
| 25 | 63.2 ± 2.5 | 13.8 ± 0.7 | 17.8 ± 0.4 | 0.22 |
| 30 | 66.7 ± 1.9 | 12.2 ± 1.0 | 15.2 ± 0.3 | 0.18 |
| 35 | 78.8 ± 4.6 | 11.8 ± 0.6 | 7.2 ± 0.0 | 0.15 |

Data represent means ± SE obtained from four independent experiments.
[a]Ratio indicates S phase divided by $G_1$ phase.

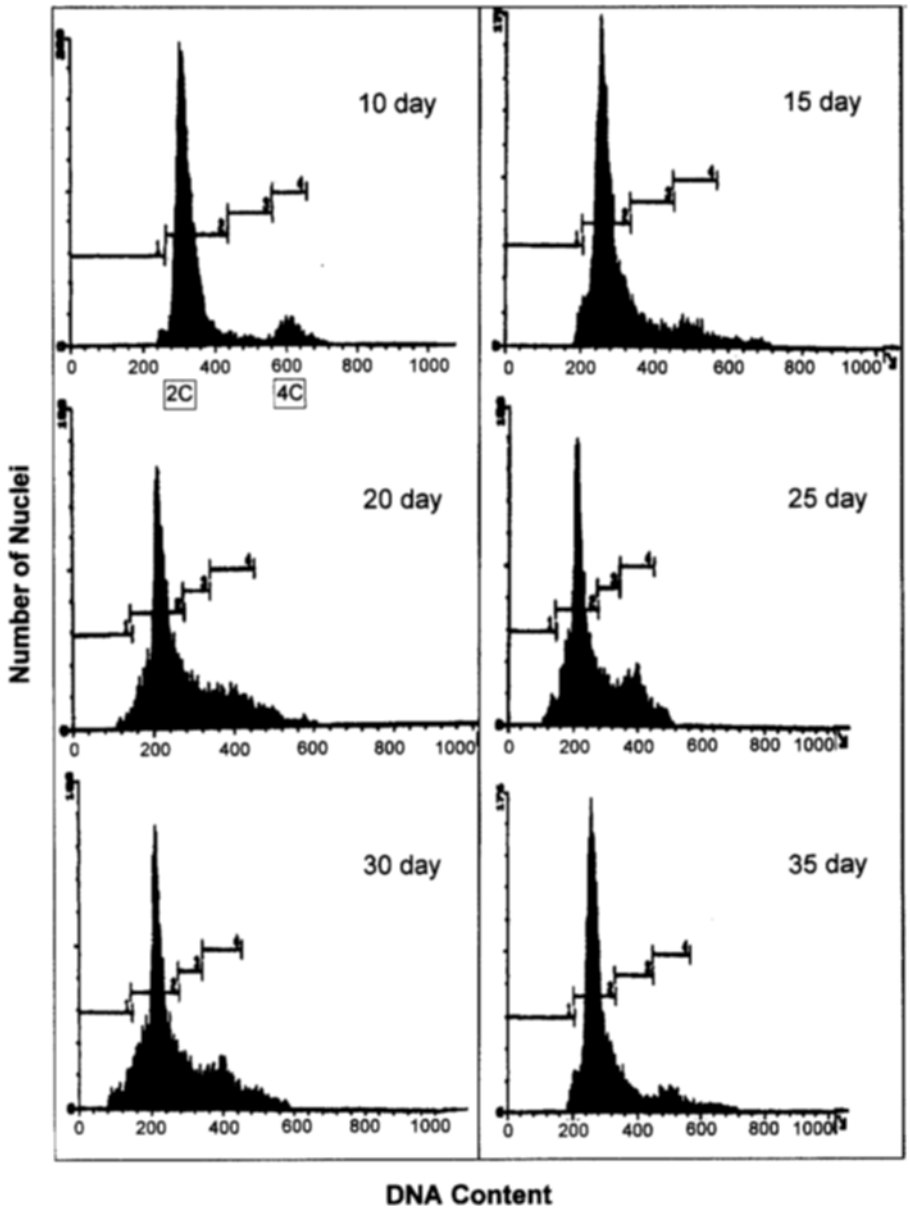


**Figure 2.** DNA contents during carrot callus culture. Contents were measured from carrot cotyledon calli cultured on MS solid media with 2,4-D (5 μM) for 10 to 35 days. 1, debris; 2, $G_1$ phase; 3, S phase; 4, $G_2$/M phase.

**Table 2.** Effect of 2,4-D concentration on somatic embryogenesis and cotyledon morphology of somatic embryos in carrot cell cultures.

| 2,4-D (μM ) | No. of somatic embryos/explant | Cotyledon shape or number (%) | | | | |
|---|---|---|---|---|---|---|
| | | Cup | 1 | 2 | 3 | ≥ 4 |
| 1 | 64.1 ± 5.2 | 20.1 ± 1.9 | 28.7 ± 1.7 | 37.3 ± 2.8 | 13.2 ± 1.2 | 0.7 ± 0.0 |
| 5 | 80.7 ± 6.1 | 17.0 ± 2.1 | 21.6 ± 1.8 | 50.7 ± 2.7 | 10.2 ± 1.4 | 0.5 ± 0.0 |
| 25 | 24.3 ± 3.2 | 29.9 ± 3.2 | 31.3 ± 4.0 | 30.0 ± 3.4 | 7.5 ± 0.9 | 1.3 ± 0.1 |

Carrot cotyledon explants were cultured for 20 days on MS solid media containing various concentrations of 2,4-D, then subcultured onto the same medium without 2,4-D for somatic embryogenesis for 35 days. Data represent means ± SD, obtained from four replicates.

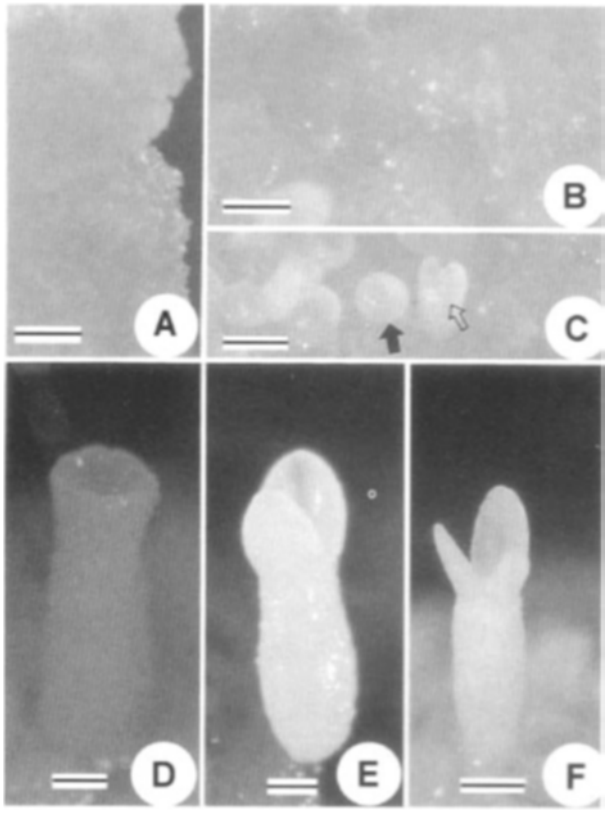


**Figure 3.** Cotyledon morphology of somatic embryos in carrot cotyledon explant cultures. Explants were cultured on MS solid media containing 5 μM 2,4-D for 20 days, then transferred onto MS solid media without 2,4-D for another 35 days. **A**, nonembryogenic callus; **B**, embryogenic callus; **C**, globular (black arrow) and heart-shaped embryos (white arrow); **D**, cup-shaped cotyledon; **E**, somatic embryo with two cotyledons; **F**, somatic embryo with three cotyledons. All bars = 400 μm.

ture. The $G_1$ phase was decreased from 95.9% at Day 5 to 63.2% at Day 25, but was increased at Day 30. The period of $G_2$/M phase increased from 1.5% at Day 5 to 17.8% at Day 25, then decreased after Day 30. Thus, the period of S and $G_2$ phases increased after the decrease in the $G_1$ phase.

Somatic embryos were derived from explant calli that was cultured on the 2,4-D-free medium; this calli had been formed on culture media containing various concentrations of 2,4-D. The amount of cell proliferation was almost directly proportional to the 2,4-D concentration (Fig. 1).

Active cell proliferation occurred from Day 15 to 30, with embryos being actively produced from Day 15 to 25. The rate of somatic embryo formation on the 2,4-D-free medium, followed by pre-culture on a medium with 5 μM 2,4-D, was high (81%; Table 2).

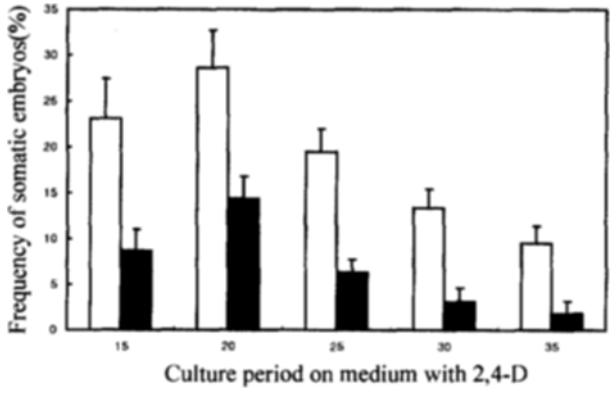


**Figure 4.** Effects of length of culture period on mature somatic embryo production from carrot cotyledon explants cultured on a medium with 5 μM 2,4-D. Frequencies of cotyledonary embryos among total embryos, and normal embryos with two cotyledons among total cotyledonary embryos were compared. The bars represent mean ± SE (n = 4). □, cotyledonary embryos; ■, dicotyledonary embryos.

For somatic embryos that had been induced on the medium with 5 μM 2,4-D, the formation frequency of normal embryos with two cotyledons was the highest compared with those from calli that had been treated with serial concentrations of 1 to 25 μM 2,4-D.

Prominently, somatic embryos with cup-shaped cotyledons were derived from cultures on media containing 25 μM 2,4-D, although such embryos were also formed after culturing with 1 μM 2,4-D (Table 2, Fig. 3). Thus, somatic embryos with abnormal cotyledons were more frequently observed after culturing on media with higher concentrations of 2,4-D.

Among all developing and developed somatic embryos, 28.6% were cotyledonary. These were derived from calli that had been pre-cultured for 20 days on a medium containing 5 μM 2,4-D. Of all the cotyledonary embryos, 14% were normal, i.e., having two cotyledons (Fig. 4). However, 25 days after culturing under the same conditions, the production rate was lower for both total and normal somatic embryos. At the cotyledonary stage, the overall production rate for somatic embryos was 20%; 6% of these were normal. After 30 days of pre-culture on a medium containing 5 μM 2,4-D, somatic embryos were produced at a very low rate.

## DISCUSSION

Based on the fresh weights of carrot cotyledon explants cultured for 50 days on media containing 5

μM 2,4-D, growth patterns followed a sigmoid shape. This also had been shown in other plant species (Wilson et al., 1971). Cell cycles in explants on the medium with 2,4-D changed from the $G_0$ into the S phase as the culture period progressed; the highest increment for the S phase was obtained at 25 days after culture. This was similar to the results from studies of seeds (Sgorbati et al., 1989) and roots of soybeans (Sparvoli et al., 1991). In the present study, the S phase decreased at Day 30, when the lowest level of cell division activity was observed. This probably resulted from nutrition consumption during the long-term culture and because of the effects of accumulated toxic compounds that were released from the cultures (Komamine, 1991).

The highest frequency of S-phase cultured cells appeared at Day 25. This was also the period for the highest frequency of somatic embryogenesis. These results suggest that explants with actively dividing cells have a high potential for somatic embryogenesis. A similar phenomenon was noted by Soh et al. (1997), who showed that somatic embryogenesis occurred more actively in shoot tip cultures, where the capacity for cell division was greater.

Over the range of tested concentrations, the production frequencies for total somatic embryos and for normal embryos with two cotyledons were high from explants cultured on the media containing 5 μM 2,4-D. Similar results had been obtained for immature zygotic embryos of soybeans cultured on a medium with 1 to 2 mg/L ($0.5\sim1 \times 10^{-5}$M) 2,4-D (Choi et al., 1994), as well as for young leaves of *A. cordata* cultured on a medium with 1 mg/L 2,4-D (Lee and Soh, 1993a, 1993b). However, somatic embryogenesis from carrot suspension cultures was highly induced on media with $5 \times 10^{-7}$M or $5 \times 10^{-8}$M 2,4-D (Fujimura and Komamine, 1980; Nomura and Komamine, 1985). In the current study, numerous somatic embryos in the globular stage were formed on a medium containing 50 μM 2,4-D. This was similar to the results obtained from soybean cultures on media with 10 mg/L 2,4-D (Choi et al., 1994). Therefore, the optimum concentration of 2,4-D for the induction of somatic embryos depends on plant species and explant type.

The most frequent somatic embryogenesis occurred when cultures were maintained on a medium with 5 μM 2,4-D for 25 days, but under those conditions somatic embryos with abnormal cotyledons increased in comparison with other types. Therefore, cotyledon morphology of somatic embryos may be affected by the duration of the 2,4-D treatment. In other studies, somatic embryogenesis, such as in young leaves of cassava, was inhibited when they were cultured on a medium with a higher concentration of 2,4-D (12 mg/L; Konan et al., 1994). Likewise, somatic embryogenesis from soybean explants on a medium containing 10 mg/L 2,4-D was arrested at the globular stage (Choi et al., 1994). Therefore, embryo development can be inhibited by a high concentration of 2,4-D on the culture medium.

The frequency of abnormal embryos with variant cotyledons also was affected by the length of the culture period. For example, greater abnormalities in cotyledon development were seen after 25 days of culture than after 20 days. A high frequency of abnormality also was reported when young leaves of *A. cordata* were cultured for five weeks on a medium containing 1 mg/L 2,4-D, without subculture (Lee and Soh, 1993a). Therefore, the appearance of abnormal somatic embryos is related to the length of time they are cultured on media with 2,4-D (Soh et al., 1996).

In conclusion, the S phase in the explants cultured on a medium with 5 μM 2,4-D was high 25 days after culture. In addition, the frequency of normal embryogenesis was higher at Day 20 than at Day 25. Thus, we could verify that active somatic embryogenesis apparently was related to cell division activity, and that somatic embryos induced from actively dividing cells were apt to accompany cotyledonary abnormality.

## ACKNOWLEDGEMENT

This study was supported in part by a research grant from the Basic Science Research Institute Program (BSRI-1998-015-D00205), Ministry of Education, Republic of Korea.

Received May 9, 2000; accepted June 26, 2000.

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