Synchronization of Somatic Embryogenesis in a Carrot Cell Suspension Culture

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ABSTRACT

Synchronization of somatic embryogenesis was achieved in a carrot (Daucus carota L. cv. “Kudodagosun”) suspension culture by sieving the initial heterogeneous cell population, by density gradient centrifugation in Ficoll solutions, and by subsequent repeated centrifugations at a low speed (50g) for a short time (5 seconds), followed by transferring the cell clusters obtained, which were composed of 3 to 10 cells, to a medium containing zeatin (0.1 micromolar) but no auxin. The frequency of embryo formation reached more than 90%, and synchrony of the embryogenetic process was observed at least in the early stages of the process. The system established in the present work provides a useful system for biochemical research into the mechanisms of somatic embryogenesis.

Little progress has been made in the physiological and biochemical investigation of embryogenesis in suspension cultures, because of the difficulty in obtaining a system in which embryogenesis occurs synchronously at a high frequency. Some attempts have been made to improve embryo formation by modifying the culture media (1, 2, 5, 7) or by making the initial cell population homogeneous (2, 3), but there has been no success in improving embryo formation to a point sufficient for biochemical research. Warren and Fowler (6) reported a method to separate globular, heart and torpedo stage embryos using glass beads to screen cultures. Their simple and effective procedure is useful for the biochemical research into the developmental processes of embryogenesis but not for the initial process of embryogenesis because embryogenesis before globular stage can not be followed by their method. In a previous paper (2), we reported that zeatin showed a marked ability to enhance embryo formation in a carrot cell suspension culture and that embryogenesis was induced at a considerably higher frequency in a medium containing zeatin but no auxin. The synchrony of embryogenesis, however, had not been achieved in the system. After many attempts to revise the previous method, we managed to achieve synchronization of embryogenesis from cell clusters of a smaller size and at a higher frequency than in previous work. The system described here provides a useful system for physiological and biochemical investigations of somatic embryogenesis.

MATERIALS AND METHODS

Plant Material and Culture Methods. The cell culture used in these experiments was initiated from a hypocotyl of a domestic carrot variety, D. carota L. cv. “Kudodagosun,” the seed of which was purchased from Kyowa-Shubyo Co. The cells were subcultured every 7 days for 6 to 12 months in a modified Lin and Staba medium (4) as described previously (2). The modification consisted of increasing the concentration of KNO₃ to 55 mM and adding 5 mM NH₄Cl instead of NH₄NO₃. Additional components of the medium were 0.1 mM FeSO₄, 0.1 mM Na₂EDTA, 9 µM thiamine·HCl, 40 µM nicotinic acid, 2.4 µM pyridoxine·HCl, 20 g/liter sucrose, and 0.5 µM 2,4-D. The medium without 2,4-D is referred to as the basic medium. The stock cell suspension culture (150 ml) was grown at 27 °C in 500-ml conical flasks on a reciprocal shaker (70 strokes/min and 5-cm amplitude) in the dark.

Induction of Embryo Formation. Embryo formation was induced by transfer of the cells to the medium containing 0.1 µM zeatin but no auxin. The density of the cell suspension was 5 x 10⁶ cell clusters/ml. The cell clusters suspended in 2 ml of the medium were rotated horizontally in a tube (18 x 180 mm) at 7 rpm in the dark at 27°C.

Counting of Cell Clusters and Embryos. The cell clusters and embryos in a culture tube were precipitated by centrifugation, and all of the precipitate was suspended in 0.4 ml of the basic medium in a counting chamber (10 x 40 x 1 mm) fixed on a slide glass. The number of all cell clusters and embryos in the chamber was counted under a microscope. The number of the globular, and heart and torpedo stage embryos was counted separately. Any embryos showing a depressed part in the surface of their otherwise globular shape were regarded as heart stage embryos. Heart and torpedo stage embryos were counted without distinction. Transparent and spherical shaped cell clusters, which were composed of smaller cells than the initial cells, were regarded as globular stage embryos.

Chemical. Ficoll was purchased from Pharmacia Fine Chemicals, Uppsala, and used after dialysis against water. Toxicity of dialyzed Ficoll was not observed in any experiments in this work.

RESULTS AND DISCUSSION

Attempts were made in this investigation to establish an experimental system in which synchronous embryogenesis occurs at a high frequency. The cell population in the stock culture was heterogeneous in its size and density (Fig. 1A). We attempted to make the cell population homogeneous first by sieving and second by density gradient centrifugation. Seven-day-old cells subcultured in the medium containing 2.4-D at the stationary phase were passed through a nylon screen with 47-µm pores and then through one with 31-µm pores. The cells and cell clusters retained on the 31-µm screen were collected. The frequency of embryo formation per inoculated cell cluster was less than 1% when embryos were induced directly from the cell population of this size. This frequency is much lower than that reported in our previous work in which cells and cell clusters in the size range 47 to 81 µm were used. However, the fraction of the size range 31 to 47 µm was used in the present work for the following two reasons. (a) It is important in the biochemical research of embryogenesis to eliminate as much as possible the dilution of biochemical events specific for embryogenesis with nondifferentiated cells. Therefore, it is desirable that the ratio of cells forming embryos to those not forming embryos in a cell
cluster be maximized. For the same reason, the cell clusters of the initial material are to be as small as possible. The initial cell clusters of the size 31 to 47 μm composed of 3 to 10 cells were found to be the smallest cell clusters in which embryogenesis could be induced. Embryogenesis occurred rarely in cell populations of sizes smaller than 31 μm. The cell clusters of the size 47 to 81 μm used in the previous work (2) were composed of 20 to 100 cells, in which the events specific for embryogenesis would be diluted with nondifferentiated cells in the cluster to a greater extent than in the cell clusters used in this work. (b) By further fractionation of this cell population as described below, synchronous embryogenesis could be obtained at high efficiency.

Serial observation of embryogenesis under a microscope showed that embryos were formed only from clusters composed of cytoplasm-rich cells, and not from those composed of vacuolated cells. The cell population of the size, 31 to 47 μm, contained many vacuolated cells and cell clusters. Therefore, further fractionation of the cell population was attempted using density gradient centrifugation.

One ml of a suspension of cells and cell clusters (packed cell volume: 0.5 ml) was layered on a Ficoll (10–18%, w/v in water, 8 ml in total volume) discontinuous density gradient in a 10-ml tube and then centrifuged at 150g for 5 min. Sucrose (2% at final concentration) was added to the Ficoll solutions to adjust the osmotic pressure. After centrifugation, fractionated cells and cell clusters on each different Ficoll concentration were suspended in the basic medium and centrifuged again at 100g for 10 s.

The total numbers of formed embryos and the frequency of embryos formed from each fraction separated by density gradient centrifugation are shown in Table I. No embryo was formed in the lighter three fractions. However, more than 90% of cells or cell clusters of the initial cell population were contained in these fractions. The highest frequency of embryo formation was observed in the heaviest fraction (>18%). Since this frequency is still not sufficient for biochemical research, a further attempt was made to increase the frequency of embryo formation.

Cell clusters in the heaviest fraction were suspended in 10 ml of the basic medium in a 10-ml tube and fractionated by repeated (five times) centrifugation at low speed (50g) for a short time (5 s). This technique provided embryos at a frequency of 47% in many clusters. The fractionation of cell clusters was repeated in the heaviest fraction 10 times as described in the previous work (2).

Table I. Fractionation of Cell Clusters by Density Gradient Centrifugation

<table>
<thead>
<tr>
<th>Fraction (Ficoll Concentration, %)</th>
<th>Total No. of Cell Clusters in the Fraction (×10⁷)</th>
<th>No. of Embryos (×10⁶)</th>
<th>Frequency of Embryo Formation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;10</td>
<td>30.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10-12</td>
<td>31.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>12-14</td>
<td>8.9</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>14-16</td>
<td>3.8</td>
<td>17.1</td>
<td>4.5</td>
</tr>
<tr>
<td>16-18</td>
<td>0.41</td>
<td>4.5</td>
<td>11.1</td>
</tr>
<tr>
<td>18&lt;</td>
<td>0.15</td>
<td>2.8</td>
<td>18.8</td>
</tr>
</tbody>
</table>

1 Formed embryos as a percentage of the initial number of cell clusters.

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**FIG. 1.** Photographs of carrot cultured cells. A: cell population of stock suspension culture 7 days after subculture. B: cell clusters obtained after fractionation by sieving with nylon screens of 31-μm and 47-μm pores, 10 to 18% Ficoll density gradient centrifugation at 150g for 5 min and repeated (five times) centrifugation at 50g for 5 s. C: culture on 7th day after transfer to the embryo-inducing medium showing many globular stage embryos. D: culture on 13th day after transfer to the embryo-inducing medium showing many heart and torpedo stage embryos. In each photograph, the bar represents 100 μm.

**FIG. 2.** Time course of embryo formation from carrot cell clusters in the auxin-free medium containing 0.1 μm zeatin. Cell clusters were fractionated by the procedure described in the text. (O): Total number of formed embryos. (●): Number of globular stage embryos. (△): number of heart and torpedo stage embryos. Bars indicate sn (N = 3).
in the culture medium. This simple procedure was found to be very effective in increasing the frequency of embryo formation by removing thoroughly the vacuolated cells still present in the fraction.

The cell clusters in the final fraction (Fig. 1B) were transferred to the embryo-inducing medium. Figure 2 shows the time course of embryo formation, indicating that globular stage embryos began to appear on the 4th day, followed by a rapid increase in the number of globular stage embryos. The number of embryos reached its maximum on the 7th day. These data indicate that the cell clusters differentiated to globular stage embryos synchronously for these 3 days. Figure 1C shows the culture on the 7th day, in which a few heart-shaped embryos in addition to globular stage embryos or undifferentiated cell clusters were observed. The total number of embryos did not change thereafter, while that of globular stage embryos decreased rapidly. On the 8th day, the first heart stage embryos were observed and the number of heart and torpedo stage embryos began to increase rapidly and reached its maximum on the 13th day. Figure 1D shows heart and torpedo stage embryos in the 13th day. The frequency of embryo formation from the initial cell clusters reached more than 90%. The results in Figure 2 indicate that the process of embryo formation was fairly well synchronized in this system, especially in the early stage of embryogenesis. The reproducibility of this system was good when cells subcultured for 6 to 12 months were used. While the criteria of synchrony were based on morphological observations, biochemical studies of the process of embryo formation should be possible using the synchronous system established in this work.

LITERATURE CITED