A Rapid Method for Transformation of Carrot (Daucus carota L.)
by Using Direct Somatic Embryogenesis

Yoshihiko Tokui and Hiroyuki Fukuda

Department of Biological Sciences, Graduate School of Science, University of Tokyo,
Hongo 7-3-1, Bunkyo-ku, Tokyo 113-0033, Japan

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Several genes that may be involved in embryogenesis have been isolated from somatic embryos of carrot by many workers. However, the function of these genes has not been discovered yet. As the first step toward finding the function of these genes, we established a rapid and efficient method for transformation of carrot by using direct embryogenesis from hypocotyl segments treated with 2,4-dichlorophenoxyacetic acid (2,4-D) for a short period.

Key words: transgenic plant; somatic embryo; carrot; Agrobacterium tumefaciens

Somatic embryogenesis of carrot is an efficient model system for the study of plant embryogenesis. Using the carrot system, various physiological and morphological features occurring during embryogenesis have been described. This system has also been used for isolation of genes that are involved in embryogenesis, including genes encoding a lipid transfer protein, an elongation factor, embryogenic proteins, a glycine-rich protein, homeodomain proteins, and a receptor-like kinase. To understand the function of such genes in embryogenesis, the transfer of the genes into embryos is desirable. Agrobacterium-mediated transformation of carrot has been reported for callus from root cortex, suspension culture from hypocotyl segments, and plantlets regenerated from somatic embryos. In this system, carrot cells were cultured in the presence of the synthetic auxin 2,4-D for a long time (1 to 3 months). Long exposure to 2,4-D for the plant tissues has been indicated to cause somaclonal variations and this is not desirable for analysis of transgenic plants.

Recently, Masuda et al. and Tokui and Masuda reported that somatic embryos were rapidly and frequently formed in the absence of endogenous auxin from epidermal cells of carrot hypocotyls when the hypocotyl had been treated with 1 mg/1,2,4-D for 24–48 h. This direct somatic embryogenesis was thought to be efficient for making a new system for gene transfer into carrot embryos, because the short exposure of 2,4-D may reduce somaclonal variation and because this system may allow the rapid production of transgenic embryos. Using this direct embryogenesis, we report here the establishment of a rapid and efficient system for transformation of carrot.

Materials and Methods

Seeds of Daucus carota L. cv. Senkou-gosun were surface-sterilized by soaking in 70% (v/v) ethanol for 5 min and then in 1% active sodium hypochlorite solution for 10 min. The sterilized seeds were placed on Murashige and Skoog (MS) solid medium with 0.2% gellan gum and 3% sucrose, and grown in the dark at 27°C. For transformation, 100 hypocotyl segments (0.5–1 cm) from 14-d-old seedlings of carrot were cultured in 20 ml of MS liquid medium with 1 mg/1,2,4-D for 48 h at 27°C with shaking on a reciprocal shaker at 85 rpm. These segments were washed in MS liquid medium without phytohormones, placed on MS solid medium without phytohormones (20 segments per 90-mm diameter plate), and cultured for 0 to 7 days.

Agrobacterium tumefaciens strain EHA101 harboring a binary vector pIG121Hm was used for transformation. pIG121Hm has a kanamycin-resistance gene, a hygromycin-resistance gene, and a 35S promoter intron-β-glucuronidase (GUS) gene in the T-DNA region. A. tumefaciens was grown in 3 ml of YEP medium (10 g/l bacto peptone, 10 g/l yeast extract, 5 g/l NaCl) with 50 mg/l hygromycin and 50 mg/l kanamycin at 27°C on a reciprocal shaker at 85 rpm for 36 h.

A hundred pieces of hypocotyl segments were incubated in 5 ml of bacterial suspension in MS liquid medium at 27°C for 2 h with shaking at 85 rpm, and then transferred onto filter paper to remove any excess of bacteria suspension. The segments (20 segments/plate) were further co-cultured with the bacteria on phytohormone-free MS solid medium for 3 to 7 days. After co-culture, these hypocotyl segments were washed with MS liquid medium (100 segments/ml) containing 250 mg/l cefotaxime for 1 h at least five times and then they were cultured on MS solid medium containing 15 mg/l hygromycin and 250 mg/l cefotaxime (20 segments/plate) in the dark at 27°C. These segments were transferred onto fresh medium containing the antibiotics every week. After 4 weeks of selection, somatic embryos resistant to hygromycin were formed on the hypocotyl segments, and numbers of embryos and hypocotyls that formed embryos were scored. Each embryo was transferred onto MS solid medium with 15 mg/l hygromycin and 250 mg/l cefotaxime and further grown at 27°C.
Expression of GUS in carrot cells was assayed essentially as described by Jefferson et al., with 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) as a substrate. Somatic embryos or hypocotyl segments of carrot were fixed in 10 mM MES buffer (pH 5.6) that contained 0.3% formaldehyde and 0.3 M mannitol for 45 min at room temperature and then washed in 50 mM sodium phosphate buffer (pH 7.0) at least 3 times. The washed samples were stained in phosphate buffer containing 1 mM X-Gluc, 0.5 mM potassium ferricyanide, and 0.5 mM potassium ferrocyanide overnight at 37°C after mild vacuum for 5 min. Stained samples were observed with a stereomicroscope (Wild M3Z). Samples for scanning electron microscopy were prepared as described by Masuda and Tokuiji and observed under scanning electron microscope (SEM) (Nihon Denshi, JSM-6301F) at 5 or 10 kV.

Results

For re-establishment of the method for efficient formation of somatic embryos, we optimized some conditions of materials and culture. As starting materials, hypocotyl segments from dark grown 14-d-old seedlings of carrot (cv. Senkou-gosun) were found to be the most efficient for direct embryogenesis. Treatment of hypocotyl segments with 1 mg/l 2,4-D for 48 h was the most suitable for embryo formation.

Next, we tried to get transgenic embryos using this direct embryogenesis. For that purpose we used A.

![Diagram](attachment:image.png)

Fig. 2. Effects of Duration of Culture before Infection with Agrobacterium on the Formation of HmR Embryos of Carrot. Values and bars indicate averages and standard deviations from three independent experiments, respectively.

![Diagram](attachment:image.png)

Fig. 3. Protocol for the Formation of Transgenic Embryos from Hypocotyl Segments of Carrot.

(A) A freshly isolated hypocotyl, (B) A hypocotyl that had been cultured for 5 days after the treatment with 2,4-D.
tumefaciens EHA101 harboring a plasmid, pIG121Hm, containing cauliflower mosaic virus 35S promoter-intron-GUS, hygromycin phosphotransferase, and neomycin phosphotransferase genes. At first we could not get any transgenic embryos if we infected carrot segments with *Agrobacterium* at the onset of

![Fig. 4. Transgenic Embryos and Plants of Carrot.](image)

(A) Transgenic embryos formed on an explant cultured on medium with hygromycin. (B) Embryos stained with X-Gluc. Note that most of embryos are stained blue. (C) A torpedo embryo stained with X-Gluc. (D) A plantlet stained with X-Gluc. (E) A transgenic plant. (F) A leaf of (E) stained with X-Gluc. (G) Roots of (E) stained with X-Gluc.
Table 1. Effects of Duration of Infection and Co-culture with *Agrobacterium* on the Formation of HmR Embryos

<table>
<thead>
<tr>
<th>Incubation with Agrobacterium (h)</th>
<th>Co-culture with Agrobacterium (d)</th>
<th>Frequency of explants with hygromycin-resistant embryos (%)</th>
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<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>1</td>
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<tr>
<td>2</td>
<td>3</td>
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<td>2</td>
<td>5</td>
<td>8</td>
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<tr>
<td>2</td>
<td>7</td>
<td>0**</td>
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* 100 hypocotyl segments were cultured for 5 d for each experiment.
** No HmR embryo was found, because of overgrowth of *Agrobacterium*.

culture of segments. Observation of cultured hypocotyls with SEM showed that the surface of epidermal cells of intact hypocotyls was covered with a cuticular layer, but the cuticular layer over epidermal cells was broken during culture after the treatment with 2,4-D (Fig. 1). We thought that this cuticular layer may make it difficult for *Agrobacterium* to invade epidermal cells that are to form somatic embryos. Therefore, we examined the optimum duration of culture after treatment with 2,4-D before infection (Fig. 2). Transgenic embryos were selected with hygromycin. No hygromycin-resistant (HmR) embryos were formed in the absence of this culture after treatment. Culture for 5 d after treatment, which caused a conspicuous break down of the cuticular layer of hypocotyl segments, resulted in the highest transformation frequency. Duration of co-culture with *Agrobacterium* was also examined from 3 to 7 d (Table 1). One-hour incubation with a suspension of *Agrobacterium* resulted in low frequency of formation of HmR embryos, even if the co-culture period was long enough. Two-hour incubation was sufficient for formation of HmR embryos. With 2 h of incubation, co-culture for 3 d was not enough for formation of a reasonable number of HmR embryos and co-culture for 7 d resulted in overgrowth of *Agrobacterium* and in failure of formation of HmR embryos. Co-cultivation for 5 d allowed a reasonably high frequency of formation of HmR embryos. Figure 3 indicates a protocol based on these results. According to this protocol, 100 and 9 HmR embryos per segment were formed at maximum and at average, respectively, in about 10% of the segments in 3 weeks (Fig. 4A). HmR callus were also formed on these segments at a much lower frequency (data not shown). GUS activity in these HmR embryos was assayed by incubating with X-Gluc (Fig. 4B, C). Almost all of these embryos were stained blue. In most of the embryos, the whole embryo was stained, but in some embryos, limited parts of embryos were stained. These embryos developed to plantlets (Fig. 4D) and then mature plants (Fig. 4E). All tissues of these plants were also stained blue with X-Gluc, showing that these are transgenic plants (Fig. 4D, F, G).

**Discussion**

In this report, we have established a method for obtaining rapidly transgenic somatic embryos in carrot. The most serious obstruction in the establishment of the method was the presence of cuticle in the epidermis. The cuticle prevented *Agrobacterium* from invading the epidermis. Wounding was reported to be effective for the infection with *Agrobacterium*, 23,24 However, in our case, wounding did not promote the infection efficiency with *Agrobacterium*, or the formation of transgenic somatic embryos. We overcame this difficulty by prolonging of the culture of hypocotyl segments, which resulted in breakdown of the cuticle. The prolonged culture, however, may bring about a chimeric embryo which is composed of both of transformed and non-transformed cells because the infection may occur after cell division of an embryogenic epidermal cell. Indeed, although most of the hygromycin-resistant embryos had GUS-activity all over the embryo, some embryos showed GUS-activity in limited areas of the embryos. This result may indicate the presence of chimeric embryos. Tokuj et al.25 have reported the formation of secondary embryos from a plantlet derived from a somatic embryo of carrot. Even if some chimeric embryos are included in transgenic embryos, we can induce real transgenic embryos from such chimeric embryos by using the method of Tokuj et al.25

The method that we have established allows us to make transgenic embryos rapidly and efficiently. Methods that had been reported so far needed fairly long culture periods for getting carrot transgenic embryo. They needed a couple of months for making embryogenic cultures of callus or suspension cells, and about a month for differentiation of embryos from the transgenic callus. 10-12 In addition, in the previous methods, cells were exposed to 2,4-D for a long period, which sometimes caused somaclonal variation in cells. 13,14 Our method can minimize the possibility of somaclonal variation, because of very short treatment with 2,4-D and direct embryogenesis from epidermal cells.

Many genes that are involved in somatic embryogenesis of carrot have been isolated. However, function of almost of these genes has not been identified. The method that we reported here may be very useful for elucidation of the function of these genes in embryogenesis. In fact, the function of homebox genes in somatic embryogenesis is being revealed in our laboratory using this method.

**References**

A Rapid Transformation of Carrot


