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Characterization of two-step direct somatic embryogenesis in carrot

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Abstract

Direct somatic embryogenesis has been used to induce intact regenerated plantlets. We applied two-step direct somatic embryogenesis to the mass propagation of carrots and characterized this method. Morphological observation revealed that somatic embryos were generated from whole segments except for the callus part. Secondarily formed collenchyma-like structures were observed in the segments after the induction culture. The somatic embryos were generated from the collenchyma-like structure. When the longer-grown hypocotyls which had less potential to generate somatic embryos were used, the collenchyma-like structure did not develop. RAPD-PCR investigation showed that the regenerated plantlets had genetic stability. These results indicated that two-step direct somatic embryogenesis was a useful method for the mass propagation of plants without losing the quality of the donor plants.
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Keywords: Direct somatic embryo; Carrot; Plant cell culture; Tissue cell culture; Genetic stability; DNA

1. Introduction

Direct somatic embryogenesis (DSE), which induces somatic embryos directly from donor plantlets, was supposed to be associated with the cytological and genetic stability of regenerated plantlets. Some research with random amplified polymorphic DNA (RAPD) has revealed genetic stability in DSE [1–3].

The DSE procedures can be categorized into two groups: one-step procedures and two-step procedures. In one-step DSE, somatic embryos are formed with a single culture process using donor tissue which has high regeneration potential, for example zygotic cotyledon [4–6]. On the other hand, two-step DSE, which consists of induction and development cultures, can be initiated from hypocotyl or leaf segments. We investigated the high productivity of regenerated plantlets in the two-step DSE system in our previous paper [7]. We succeeded in inducing 1700 carrot plantlets from a hypocotyl. This number was much larger than the reported values for one-step DSE in various plant species.

The induction culture in two-step DSE includes an auxin treatment similar to somatic embryogenesis via callus cells. The difference between the procedures of the two-step DSE and callus-mediated somatic embryogenesis is merely the cultivation period with auxins. Tokuji et al. also reported DSE in the carrot [8–10]. They were able to induce the direct somatic embryo with a short time exposure to a high concentration of 2,4-dichlorophenoxy acetic acid (2,4-D) within 24–48 h [9]. In this treatment, the donor plantlet kept its initial shape, and the somatic embryos were generated directly from epidermal cells. On the other hand, in our study induction culture was carried out for 2 weeks. This induction culture expanded the donor plantlet and increased the number of regenerated plantlets.

If the induction culture were prolonged, somatic embryos might be generated from the callus rather than from donor plant tissues. Also, long exposure of the plant tissues to 2,4-D was reported to cause somatic variations [11–13]. Therefore, prior to applying the two-step DSE, it should be confirmed whether the somatic embryos were generated from origins other than callus cells and whether somatic variation was suppressed during the induction culture.

The present article is concerned with the quality of this system as a propagation system. We carried out histological observation and analysis of RAPD in two-step DSE using carrot hypocotyls.

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2. Materials and methods

2.1. Cells and medium

Seeds of carrot (*Daucus carota* L. cv. Natsumaki-Senkou-gosun) were surface-sterilized by soaking in 70% (v/v) ethanol for 3 min and then in 1% active sodium hypochlorite solution for 30 min. They were washed extensively in sterile water and then allowed to germinate for 1–3 weeks at 26 °C in darkness on Murashige and Skoog (MS) solid medium with 8 g L^{-1} of agar without sucrose. Hypocotyl explants without apical meristems were removed from the seedlings and cut into 0.5 cm segments.

The hypocotyl segments were placed on MS solid medium with 0.5 mg L^{-1} of 2,4-D, 30 g L^{-1} of sucrose and 8 g L^{-1} of agar. They were incubated at 26 °C under continuous light of 2000 Lx (induction culture). After 14 days, the segments were transferred to new MS solid medium without 2,4-D and incubated for another 30 days (development culture).

2.2. Histological observation

Morphological development in the course of the cultures was observed under a stereo microscope equipped with a digital camera on the torinocular port. Thin-sectioned segment samples were observed under a differential interference microscope for histological observation.

Samples used for thin sectioning were fixed with 7.2% formaldehyde in 50 mM sodium phosphate buffer with 0.1% IGEPAL CA-630 (MP Biomedicals, Inc.) and 10% dimethyl sulfoxide for 40 min at room temperature. They were embedded in O.C.T. (Optical Cutting Temperature) compound (Tissue Tek) and frozen in liquid nitrogen. Frozen sections of 5-μm thickness were prepared using a cryostat (Leica CM1850, Nussloch, Germany). The sections were placed on slide glasses shredded with 3% low-melting temperature agarose.

The segments were fixed with a mixture of 38% formaldehyde solution, acetic acid and 70% ethanol (1:1:18), and freeze sec-

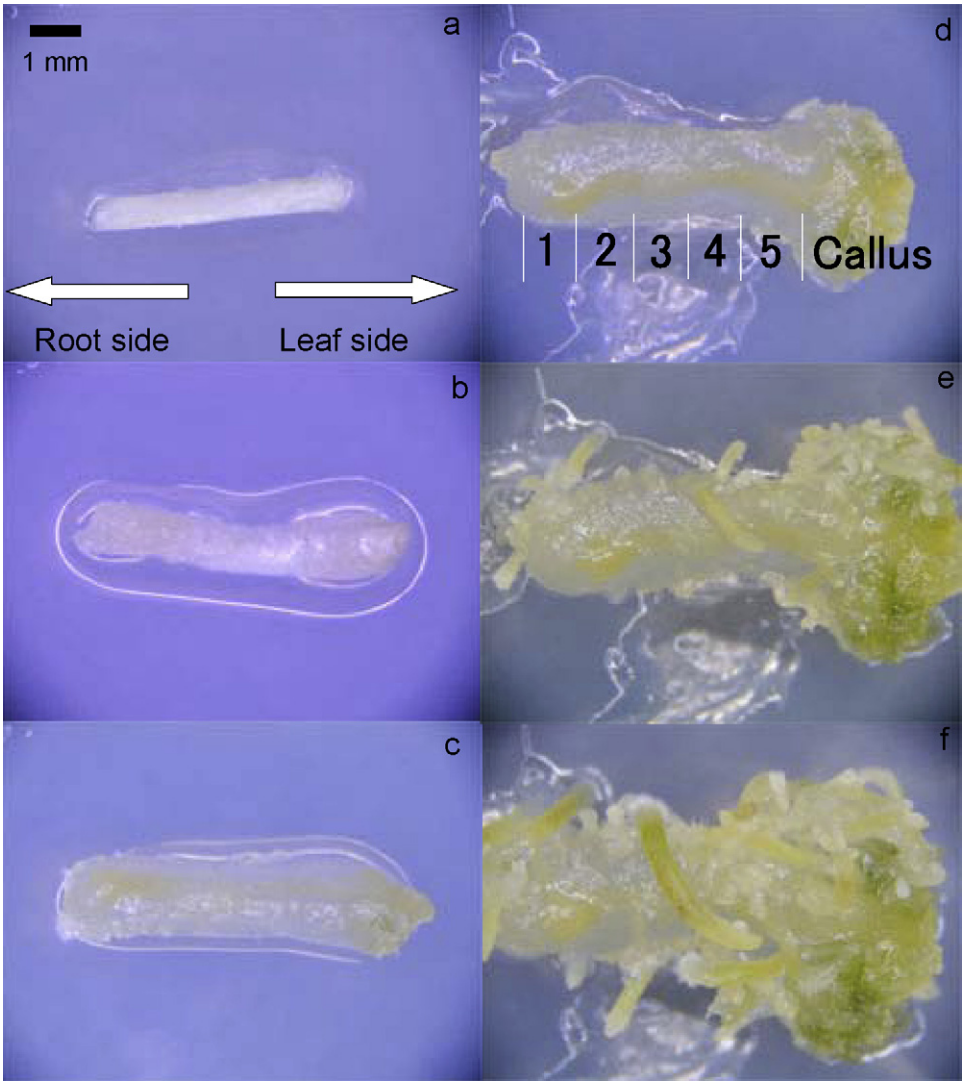

Fig. 1. Typical morphological change in a hypocotyl segment during two-step DSE. A segment was incubated for 44 days, which included the first 14 days of the induction culture and the following 30 days of the development culture. The culture periods of the shown segment were 0 days (a), 7 days (b), 14 days (c), 21 days (d), 25 days (e) and 28 days (f). The numbers in (d) represent the indices for estimating the dependency of somatic embryogenesis on the location in a segment, as shown in Fig. 2.

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