Observation of Early Stage of Somatic Embryogenesis from Epidermal Cells of Carrot Hypocotyls by Scanning Electron Microscopy

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Somatic embryogenesis that occurred directly from carrot epidermal cells was monitored by scanning electron microscopy. Epidermal cells peeled off from hypocotyl tissues were visible as compressed cells, which expanded to become tubular cells. Subsequently, tubular cells divided regularly and "horizontally" to form linear clusters of cells and then these clusters of cells divided "vertically" to begin development. Swelling structures were formed by irregular and oblique division at sites on the linear clusters of cells and these structures continued cell division to form small proembryos. Some cells at the surface of the swelling structures and proembryos expanded in every direction to cover the surface of each structure. These expanding cells appeared to form a surface layer and then cell layers piled up one another as the swelling proceeded to a globular-stage embryo via a small proembryo.

Key words: carrot hypocotyl; embryogenesis; epidermal cells; expanding cells; scanning electron microscopy

Differentiation to somatic embryos occurs directly in situ in many plant species, without a requirement for the intervening development of callus. Many researchers have studied the way in which such embryos arise directly from organized cells of plant tissues. The embryo-forming cells have common features that are characteristic of rapidly dividing meristematic tissues. Maheswaran and Williams reported that somatic embryos arise directly via budding from both single cells and multiple cells of the epidermis of primary zygotic embryos of Trifolium repens. Furthermore, they showed by histological analysis that embryogenesis was initiated by the irregular, periclinal, and oblique division of epidermal cells. We reported recently that, when hypocotyls were cultured in hormone-free medium after a brief treatment with 2,4-D, the organized epidermal cells of carrot hypocotyl explants were effectively separated from parent organs and developed directly into somatic embryos. We anticipated that, in our system, details of the embryogenic cells at each stage of the differentiation pathway from epidermal cells would be visible by scanning electron microscopy (SEM), as observed by many researchers.

We present here SEM images of the sequence of divisions that results in production of embryos from epidermal cells and the shapes of cells on the expanding surface of the embryo.

Materials and Methods

Plant materials and culture conditions. Carrot (Daucus carota L. cv. Koshingosan) seeds were surface-sterilized in 70% ethanol under a vacuum and then in 5% sodium hypochloride for 10 min. They were washed extensively in sterile water and then allowed to germinate for about 2 weeks at 26°C in darkness on basal Murashige and Skoog (MS) medium that had been solidified with 0.2% Gelrite. The hypocotyl explants without apical meristems were removed from the seedlings and cut into 1-cm pieces. Hypocotyl explants were placed in 50-ml Erlenmeyer flasks with 20 ml of liquid MS medium with 2,4-D (1.0 mg/liter) and sucrose (3% w/v), and they were cultured with agitation on a reciprocal shaker at 85 rpm in the light at 26°C. After 24 h, explants were transferred into a MS liquid

Abbreviation: 2,4-D, 2,4-dichlorophenoxyacetic acid.

Fig. 1. Separation of Epidermal Layer from Hypocotyl Explant and Epidermal Cell Division. A: Separation of epidermal layer from hypocotyl explant; ep, epidermal layer; co, cortex. B: Simultaneous observation of compressed cell, tubular cell, and dividing cell. c, compressed cells; t, tubular cells; d, cell-division region.
medium without 2,4-D and culture was continued.

Light microscopy. Embryos were fixed in 2% glutaraldehyde in 0.1M phosphate buffer (pH 7.4) at 4°C for 4h and subsequently postfixed in 1% osmium tetroxide in the same buffer for 3h. Dehydration was effected in a graded ethanol series and completed by immersion in propylene oxide. The samples were embedded in Spurr's epoxy resin, sectioned at 1 μm, and stained with toluidine blue.

Scanning electron microscopy. At each stage of culture in MS liquid medium without 2,4-D following an initial treatment with 2,4-D for 24h, hypocotyl explants and embryos were harvested for SEM studies. Samples were fixed in 2% glutaraldehyde at 4°C for 4h and then in 5% KMnO₄ overnight at 4°C, and they were dehydrated in a graded ethanol series. Explants dehydrated in 100% ethanol were then treated with tert-butyl alcohol and dried by the critical-point method. Samples for SEM were covered with a thin layer of gold and observed under a scanning electron microscope (Nikon Densi, JSM-6301F) at 5 or 10kV.

Results and Discussion
The SEM images of carrot tissue presented here, as well as previous light-microscopic images¹⁰ showed that the peeled-off epidermal cells proliferated to form somatic embryos directly via cell clusters without an intervening stage in suspension culture (Figs. 1, 2, and 4), when carrot hypocotyl explants were cultured in hormone-free medium after a brief treatment with 2,4-D. The epidermal tissues were observed to peel off from the parent hypocotyl explants (Fig. 1A), as described in our previous paper.¹⁰ The epidermal cells that appeared to be compressed by nature and then to expand to become tubular cells seem to divide, since such expansion of compressed cells has likely occurred in view of the simultaneous observation of compressed cells, tubular cells, and dividing cells as shown in Fig. 1B. Thus compressed epidermal cells did not divide until they became tubular cells. The expanded tubular cells divided regularly and "horizontally" to form linear cell clusters (Fig. 2A). Subsequently, these clusters of cells divided "vertically" to begin development. The development of cell clusters was accompanied by the appearance of cell clusters with a smooth surface as a result of the rupture of membrane-like structures that covered the epidermal tubular cells (Fig. 2B).

What is the earliest detectable morphological events in somatic embryogenesis from linear clusters of cells? The division sequences, typified by the images in Fig. 3, suggest the initiation of somatic embryogenesis from clusters of cells. Slight swellings were observed in central (Fig. 3A) and terminal regions (Fig. 3B) of clusters of cells as a result of both irregular and oblique division and, subsequently, such divisions gave rise to small globular proembryos (Fig. 4B). Subsequently, these small globular proembryos developed into globular embryos on the epidermal tissues of parent explants.¹⁰ Then globular embryos developed, in turn, into

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Fig. 2. Formation of Clusters of Cells.
A: Regular and "horizontal" division (small arrowhead) of tubular epidermal cells and the subsequent development of clusters of cells by additional "vertical" division (large arrowhead). B: Rupture of membrane structures that cover epidermal cells. Small and large arrowheads indicate clusters of cell with a smooth surface and a membrane cover, respectively.

Fig. 3. Formation of Swelling Structures at Central and Terminal Regions of Clusters of Cells.
A: Central region. B: Terminal region. The arrowheads indicate the irregular and oblique division of cells that form clusters of cells.
heart- and torpedo-shaped embryos and regenerated to plantlets.\textsuperscript{10} Linear clusters of cells were randomly studded with such swellings and small globular proembryos as shown in Fig. 4A. Most of the individual cells in cell clusters might have the capacity to form embryos under suitable conditions. If this hypothesis is correct, the first division of an epidermal cell might result in initiation of the morphogenetic pathway to a somatic embryo. Each proembryo appeared to be produced from a few adjacent cells that formed a cluster of cells. The first visible modification of the embryogenic cells appeared to be a transition from regular and longitudinal division to irregular and oblique division. This observation is in agreement with that of Maheswaran and Williams,\textsuperscript{21} who described the first signs of the induction of embryos from young epidermal cells of immature zygotic embryos of \textit{Trifolium repens}.

How do the swelling structures on clusters of cells become developed to form large globular-shaped embryos \textit{in situ} formation of small proembryos? We observed expanding cells that appeared to be involved in the development of swelling structures and of each type of embryos. Some of the superficial cells on swelling structures were first pushed outwards by longitudinal elongation and then they expanded over the surface in every direction (Figs. 5A, B, C).

This was also the case for globular-shaped embryos (Figs. 6A, B, C). Direction of cell expansion was judged from the wrinkle pattern that was present on superficial cells on swelling structures and globular embryos, that is, the wrinkle pattern of cells that had ceased expansion was coarse and that of cells that were expanding was fine. The expanding cells began to divide and the terminal regions of the structures continued to expand. Such expanding cells were randomly distributed on the swelling structures and

**Fig. 4.** Swelling Structures and Proembryos on Cluster of Epidermal Cells.
A: Swelling structures and proembryos on cluster of cells. The arrows and arrowheads indicate swelling structures and proembryos, respectively. B: Formation of a proembryo in the center region of a cluster of cells.

**Fig. 5.** Expanding Cells on the Surfaces of Swelling Structures and Proembryos.
globular-shaped embryos (Figs. 5 and 6) and they appeared to cease to expand when they encountered one another on the smooth surface of globular embryos (Fig. 7). Notably, covering of the surface of a globular embryo with the expanding cells was observed to proceed (Fig. 6C). In this way, these expanding cells probably formed a layer on the surface of the globular embryo and furthermore, the number of layers increased to enlarge the embryo. In addition, these expanding cells filled up small and large hollows on the surface of each structure, modifying the surface until it had a uniform appearance (Fig. 6C as an example).

**Fig. 6.** Expanding Cells on the Surface of Globular-stage Embryos.
A and B: Expanding cells. C: Covering of expanding cells on surface of globular embryos. Arrowhead indicates the hollow on the surface of embryo. Arrow indicates directions of elongation of expanding cells.

**Fig. 7.** Smooth Surface of Globular Embryo.

**Fig. 8.** Photomicrographs of Sectioned Proembryos and Globular Embryos.
A: Proembryos. B: Globular embryos at the early stage. C: The middle stage. D: The late stage. Arrowheads indicate protruding cells that are expanded and divided.
Light microscopy of sections of proembryos and embryos at early and late globular stage showed that the surface of the embryos was irregular, with cells that appeared to have expanded, divided, and formed a layer (Fig. 8). Also the pushed and expanded cells seemed to be present on the relative irregular surface of the embryos in late globular and early heart stages initiated from mechanically wounded mature zygotic embryos of carrot as our judgment by photomicrographs of sectioned cultures. Thus, these facts suggest that layers of cells are piled up on one another by the action of these expanding cells to form globular-stage embryos, via the small proembryos generated from the swelling structures. Furthermore, somatic embryos in late heart stage became composed of many relatively regular layers. Therefore, these expanding cells seemed to modify the surface of globular embryos to a uniform appearance of heart-shaped embryos. Dubois et al. described how the surface of somatic embryos derived directly from leaves of Cichorium was “wrinkled”. These cells that made up the wrinkled surfaces of embryos might be the expanding cells presented here. In addition, Xu and Bewley reported that the surface of alfalfa somatic embryos was very rough compared to that of zygotic embryos from observation with SEM. The protuberances observed on the rough surface of these alfalfa embryos also resemble the expanding cells presented here. Yadegari et al. demonstrated that the surface of zygotic embryos of Arabidopsis at the late-globular stage was relatively smooth, but the embryos of T-DNA-mutagenized lines contained a mosaic of irregularly sized protuberances projecting outward from their surfaces and remained globular. By contrast, though the embryos derived from epidermal cells in our embryo-forming system contained irregularly sized protuberances on their surface, they could develop into plantlets via heart- and torpedo-shaped embryos. Somatic embryogenesis in our embryo-forming system might differ from zygotic embryogenesis.

References