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Anatomical Observation of Somatic Embryogenesis in *Oenanthe javanica* (Bl.) DC.

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미나리 體細胞 胚發生過程의 解剖學的 觀察

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This experiment was carried out to observe the origin and developmental pattern of somatic embryos of *Oenanthe javanica* (Bl.) DC. The experiment included observation of embryogenic cells and their developmental stages by light microscope, transmission electron microscope and scanning electron microscope. The embryogenic cells, which were smaller than non-embryogenic cells in size with expanded nucleus and dense cytoplasm. When stained with hematoxylin, the embryogenic cells were readily distinguished from the non-embryogenic cells of which cell walls were stained with safranin. It was observed that somatic embryos developed from single cells on the epidermis of developing embryos or in the surface or inside of embryogenic clumps by segmentation pattern. Observation with a transmission electron microscope revealed that the embryogenic cells had dense cytoplasm, expanded nucleus, small vacuoles, large amyloplasts containing starch grains, and abundant organelles including lipid bodies. Under a scanning electron microscope, embryogenic callus was shown to consist of very smaller cells than non-embryogenic cells in an orderly arrangement and covered with a net-like structure, while the non-embryogenic callus consisted of large cells, irregular in size and arrangement, and covered with a gelatin-like material.

Key words: scanning electron microscope, transmission electron microscope

There are several reports on the origin and developmental pattern of somatic embryogenesis of plant in vitro. When plant tissues were cultured in vitro, certain cells in the explants or callus induced from them developed into embryos. The developmental pattern of somatic embryos resembles that of zygotic embryos. (Street and Withers, 1974). Numerous reports on somatic embryogenesis suggest that somatic embryos are derived from single cells having specific characteristics such as large nucleus, dense cytoplasm, small vacuoles and numerous cell organelles (Konar et al., 1972). To understand the process of embryogenesis precisely and to get a clear picture of the early developmental sequence, it is necessary to observe the changes taking place at the cellular or subcellular level. Some structural observations show that the embryogenic cells destined to develop into embryos are highly cytoplasmic and possess a large and diffusely stained nucleus with a single darkly stained nucleolus (Street and Withers, 1974). This study was carried out to observe the origin of embryogenesis and its developmental pattern in *Oenanthe javanica* (Bl.) DC., a Korean traditional vegetable crop.

MATERIALS AND METHODS

The calluses and clumps used for anatomical study were obtained by subculture of embryogenic callus derived from in vitro petiole segments of *Oenanthe javanica* (Bl.) DC. For the observation under light microscope, the samples were fixed in FAA solution for longer than 24 hours and then

dehydrated by passing them through n-buthyl alcohol series. After dehydration, tissues were embedded in paraplast or hard paraffin and sectioned at 5-7 μm thick. The procedure of staining and mounting followed the method described by Berlyn and Miksche (1976).

For the observation under transmission electron microscope (TEM) (Zeiss, EM 109R), the embryogenic clumps obtained from the culture of embryogenic callus in MS liquid media containing 2,4-D, NAA, IBA or NAA, and those cultured on MS basal medium were used. Specimens were prefixed in 0.2 M sodium phosphate buffer (pH 6.8) containing glutaraldehyde at 1.5% for 30 minutes at room temperature. The remaining procedure followed the method of Fowke (1984).

For the observation of embryogenic and non-embryogenic cells under scanning electron microscope (SEM), embryogenic calli which were derived from subculture of embryogenic callus on the solid media containing 2,4-D were used. The callus were prefixed in the solution of 1% glutaraldehyde and 2% para-formaldehyde for 2 hours, and then washed three times with 0.2 M sodium phosphate buffer for longer than 2 hours. Post-fixation was performed by incubating the specimens in a buffer solution of 1% OsO_4 for 2 hours. After post-fixation, the specimens were dehydrated through ethanol series, and treated by iso-amyl acetate. After drying up to critical point, they were covered with gold and observed under SEM (ZSM 330-409, Nikon Electric Co.).

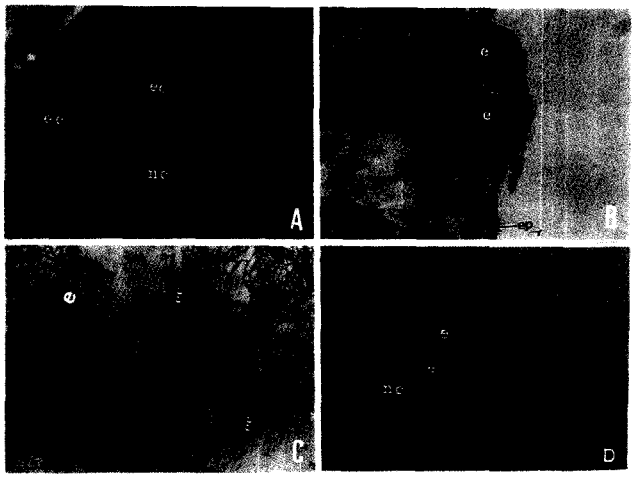

Figure 1. Embryogenic cell clumps and embryos developing from embryogenic callus cultured in suspension with media free of growth regulators. A: A layer of embryogenic cells (ec) on the embryogenic cell clumps (100x). B: Adventive embryogenesis on the epidermis (ep) of a somatic embryo. The epidermis consisted of 1 ~ 4 layers of cells, embryogenesis occurred only from certain cells (e) stained by hematoxylin in the outermost layer (1,000x). C: Embryo development in embryogenic cell clump. Embryogenic cells and pro-embryos (e) were stained by hematoxylin, but advanced embryos (ae) and non-embryogenic tissue (nc) were stained by safranin. D: Embryogenic cells (e) embedded in a large aggregate of non-embryogenic cells.

RESULTS AND DISCUSSION

Observation under Light Microscope

In the sections of the embryogenic cell clumps double-stained with hematoxylin and safranin, embryogenic cells and embryos at the early developmental stages were stained dark purple by hematoxylin, meanwhile the aged cells and non-embryogenic cells were stained light red by safranin. The hematoxylin stained cytoplasm and nuclei, while safranin stained amyloplasts, cell wall and senescent cells (Figure 1A - D). Therefore double staining with hematoxylin and safranin was considered to be an effective means to distinguish the embryogenic cells and non embryogenic cells of *O. javanica* (Bl.) DC. Konar et al. (1972) used toluidine blue or periodic acid Schiff (PAS) to stain embryogenic cells, where embryogenic and non-embryogenic cells were stained light and dark, respectively. The embryogenic cells, when double-stained by hematoxylin and safranin, were readily distinguished from the non-embryogenic ones by the color.

The embryogenic cell clumps in liquid media consisted of non-embryogenic cells, embryogenic cells and developing embryos. Embryogenic cells generally occurred in the epidermis of the somatic embryos and in the surface of embryogenic cell clumps (Figure 1A, B). The embryogenic single cells and somatic embryos were sometimes observed to be embedded within the cell clumps (Figure 1C, D). It was reported that embryogenic cells of carrot were scattered singly or in small groups among the typical mature epidermal cells, and they were smaller in size, rich in cytoplasm and contained a large nuclei (Konar et al., 1972). It was also reported that cell clumps cultured in suspension cultures of wild carrot had two histological regions; the inner region of highly vacuolated and non-dividing cells, and the peripheral region of small meristemmatic cells (Halperin and Jensen, 1967).

The embryogenic single cells in suspension culture when observed under an inverted microscope did not reveal whether to develop directly into embryos or not. Instead, these cells firstly formed embryogenic clumps, and then somatic embryogenesis (globular stage) occurred in the embryogenic

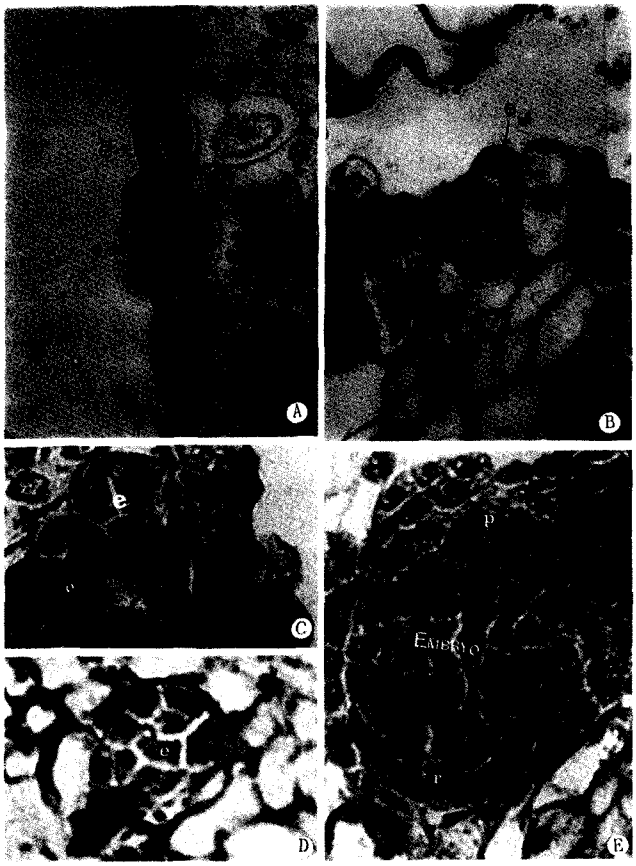

Figure 2. Embryo development in the embryogenic cell clumps of *O. javanica* (BL.) DC. A: Embryo development from embryogenic single cells (e) upto 4-cell stage (e) through segmentation. B, C: A pro-embryo (e) at more advanced stage. D: An embryo at 10-20 cell stage (e). E: A globular embryo showing plumule (p) and radicle part (r).

clumps (Figure 2A/E). The first division of embryogenic single cell occurred on the epidermis of existing embryos, the surface or inside of the embryogenic clumps. The embryogenic cells in the outermost layer of epidermis (Figure 2A) or within the embryogenic clumps divided into two daughter cells. One of the two cells divided longitudinally in the parallel with the direction of the first division (Figure 2A, C). This division products developed into plumule part, while the other cell into suspensor. This segmentation pattern was not always identical in the cleavage of the embryogenic cells. Division patterns of original cells to form globular embryos varied as shown in Figure 2A-E. The developing embryos at 8-9 cell stage clearly showed the plumule and radicle part (Figure 2D).

It was reported that the earliest cell divisions in the embryogenically determined cells can apparently follow a number of patterns without affecting the final outcome of embryogeny (McWilliam et al., 1974) and the morphogenetic

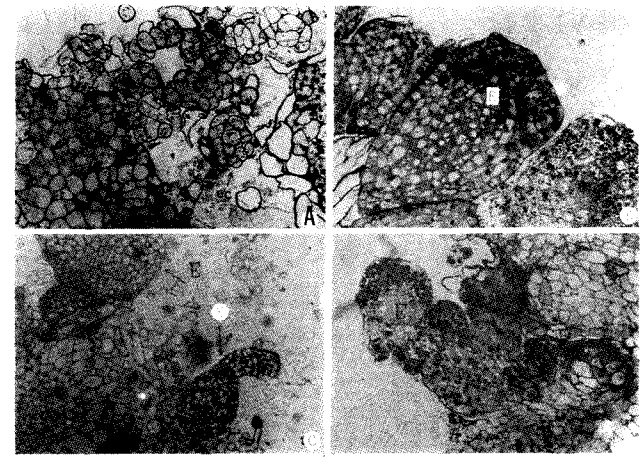

Figure 3. Embryo development from a embryogenic cell clump of *O. javanica* (BL.) DC. cultured in the liquid media containing 2,4-D, NAA, IBA and BA. A: Aggregates of embryogenic cells (ec) formed in the media containing 1 mg/L 2,4-D. B: Embryo development (E) in the medium containing 1 mg/L NAA. C: Development of embryo (E) having special appendage like suspensor (s) in the media containing 1 mg/L IBA. D: Embryo development in the medium containing 1 mg/L BA.

sequence could also be substantionally altered to result somatic embryos strikingly dissimilar or similar to zygotic embryos (Ammirato, 1987). It was reported also that somatic embryos were regenerated from single cells through in vitro culture (Kato and Takeuchi, 1963; Halperin and Wetherall, 1964; Backs-Husemann and Reinert, 1970).

When 2,4-D was added to suspension culture, a great number of embryogenic single cells formed in the periphery of embryogenic clumps (Figure 3A). The embryogenic cells did not preceded embryogenesis but existed only as embryogenic cell aggregates. The surface of the embryogenic cell clumps in the suspension culture containing 2,4-D was characteristically spherical and smooth. These embryogenic callus or cell clumps did not show any polarity or specific segmentation pattern, indicating the embryogenesis has not initiated yet (Figure 3A).

In suspension culture, embryogenic cells were founded in the media containing NAA less than in those containing 2,4-D (Figure 3B). The embryogenic cells were embedded inside or located the surface of the embryogenic clumps. Embryogenic cells and cell clumps cultured in the media containing NAA less than 0.5 m/L continued growth and development into torpedo-stage embryos. Embryos developed in these media had suspensors not well developed.

Pro-embryos in the media containing IBA also continued to grow into torpedo stage embryos and then into mature embryos. The original cells of somatic embryos occurred at

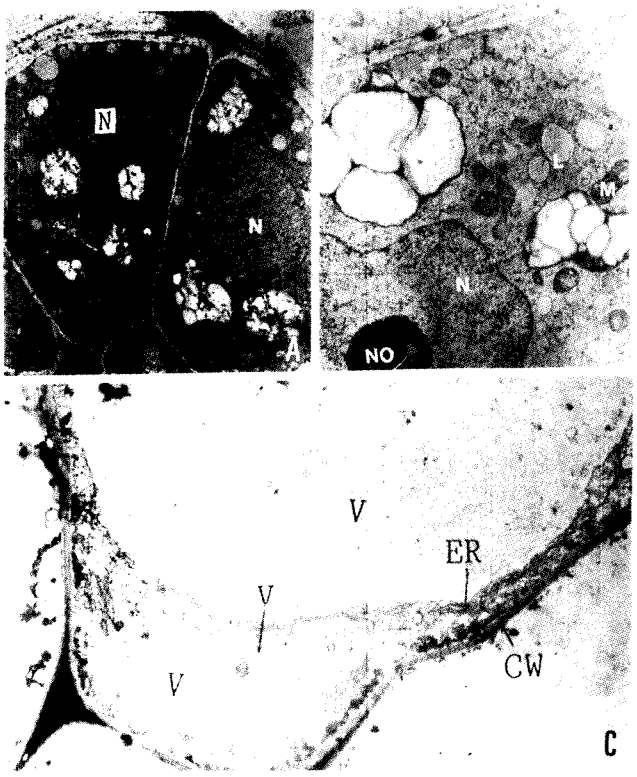

Figure 4. Transmission electron micrographs of *O. javanica* (BL.) DC. embryogenic cells cultured in the medium containing 2,4-D (1 mg/L). A: An aggregate of 4 embryogenic cells having large nuclear, dense cytoplasm, and small vacuoles (3,000x). C: Embryogenic cell showing mitochondria (M), starch grains (S), lipid body (L), a large nucleus (N) and a nucleolus (NO) (30,000x). C: A non-embryogenic cell having large vacuoles (V), cell wall (CW) and endoplasmic reticulum (ER) (3,000x).

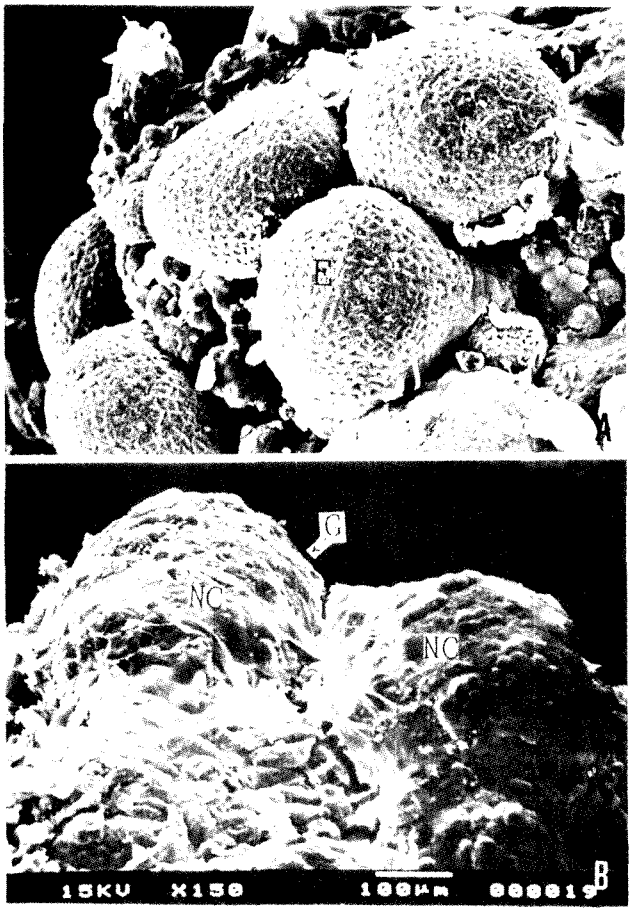

Figure 5. Scanning electron micrographs of the embryogenic and non-embryogenic callus of *O. javanica* (BL.) DC. cultured on the solid media containing 1 mg/l 2,4-D. A: The embryogenic callus consisting of developing embryos on the solid media. NC show the non-embryogenic cells derived from embryogenic callus (E) (200x). B: Non-embryogenic callus (NC) and gelatin like substance (G) on the surface (150x).

the surface of cell clumps, but somatic embryogenesis preceded less frequently on these media compared to that on the media containing 2,4-D or NAA. Somatic embryos developed in the media containing IBA or NAA had well developed suspensor-like structures (Figure 3C). Meanwhile, the suspensor-like bodies were hardly observed in the media devoid of NAA or IBA. Therefore, it was indicated that NAA or IBA promoted the growth and development of suspensor-like structures in somatic embryos. Developing suspensor-like structures in somatic embryos were readily observed in the early 5-6 cell stages of somatic embryogenesis (Figure 2C, D). Some of the suspensor-like structures degenerated as the somatic embryos developed, but others divided up to 30-50 cells on the media containing 0.5 mg/L IBA (Figure 3C).

Observation under Electron Microscope

The embryogenic cell clumps in suspension culture with the media containing 2,4-D were prepared for observation of the embryogenic single cells under TEM. The cells in the periphery of the clumps were morphologically very uniform (Figure 4A). An aggregate of 4 cells which formed on an embryogenic clump had spherical and smooth surface, but segmentation pattern of the individual cells was not shown. This cell aggregate seemed to be daughter embryogenic cells derived from one embryogenic single cell. As shown in Figure 4A, the cell wall between individual cells was very thin, but the peripheral wall was very thick. Especially cell wall between the embryogenic aggregates and neighbouring cells were very thick compared to other part of cell walls in the callus. These cells were rich in cytoplasm and clearly distinguished by corresponding structures from non-embryogenic cells or the cells in developing embryos. All the embryogenic cells were rich in amyloplasts in which several

large starch grains occurred. There was no granal organization in the stroma of amyloplasts. The nucleus was very large and round, and surrounded by numerous small vacuoles, amyloplasts and mitochondria (Figure 4A, B). There were one to two large nucleoli in the center of the nucleus. On the other hand, non-embryogenic cells were very large in size, and contained large vacuoles in cytoplasm (Figure 4C).

It was reported that the embryogenic cells destined to develop into embryos are highly cytoplasmic and possess a large and diffusely stained nucleus with a single darkly stained nucleolus (Street and Withers, 1974; Konar et al, 1972). Thus, in *O. javanica* (Bl.) DC., the pattern and content of embryogenic callus were similar to the above report.

Embryogenic callus and non-embryogenic callus observed by scanning electron microscope were quite different in shape. Non-embryogenic callus consisted of the cells irregular in size and shape, sometimes they were covered with a material like gelatin. However, embryogenic cells were uniform in size and arrangement. Their surface appeared a ball covered with a net. The surface of embryogenic callus was similar to that of an embryo (Figure 5A, B).

摘 要

미나리의 體細胞 胚發生 過程을 解剖學的으로 究明하기 위하여 胚發生 起源細胞와 캘러스를 光學顯微鏡 및 電子顯微鏡으로 觀察한 結果, 胚發生 細胞는 hematoxylin에 짙은 보라색으로, 非胚發生 細胞는 safranin에 赤色으로 染色되어 光學顯微鏡下에서 쉽게 區別할 수 있었다. 胚發生 캘러스는 많은 수의 原胚 및 發育中인 胚, 非胚發生 細胞 등으로 構成되어 있었다. 體細胞 胚發生은 發育 中인 胚나 細胞塊의 表皮細胞에 위치한 胚發生 細胞의 하나가 分裂하거나 細胞塊內의 非胚發生 細胞속에 묻혀 있는 胚發生 細胞가 分裂하여 일어났다. 胚發生 過程은 항상 一定한 形態는 아니지만 單細胞로부터 일정한 segmentation 過程를 거쳐서 胚發生이 進行되는 것으로 나타났다. 透過電子顯微鏡에 의한 觀察에서 胚發生 細胞는 非胚發生 細胞에 비하여 細胞質이 稠密하고 核이 大型이며 amyloplast, 燐脂質體 및 細胞小器官들이 많으며 液胞가 없거나 매우 작았다. 이들 細胞들은 두터운 細胞壁에 의하여 주위의 非胚發生 細胞와 分離되어 있으며 細胞輪廓은 둥글었다. 走査電子顯微鏡으로 觀察한 胚發生캘러스는 外部가 그물이 씌워진 形態의 球形의 多樣한 크기의 胚들과 比較的 크기가 큰 非胚發生 細胞들이 혼재하였다. 한편 非胚發生能 캘러스는 構成細胞가 크고 外部에는 gelatin같은 物質로 덮여 있었다.

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