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Changes in Surface Structure during Direct Somatic Embryogenesis in Rice Scutellum Observed by Scanning Electron Microscopy

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Abstract : Direct somatic embryogenesis in rice scutella was studied by scanning electron microscopy paying attention to the morphological change of surface structure. Somatic embryos were formed when the scutella of immature embryos of cv. Nipponbare were cultured on MS media containing 2 mg L^{-1} 2,4-D. Embryos were then transferred to the media containing 1 mg L^{-1} 2,4-D and germinated on hormone-free media. The developmental stages of typical monocotyledonous somatic embryo were classified into proembryo, globular, scutellar and coleoptilar stages by referring to the terminology used for zygotic embryos. Each stage had a characteristic surface structure. The surface of proembryo was smooth. Fibrillar material was observed during the transition from proembryo to globular stage. Fibrillar material was then gradually replaced by a mesh-like structure at the globular stage. With subsequent development of globular embryo, the apical scutellum region became flattened and the ridge of mesh-like structure became lower. Development of scutellum followed by the emergence of coleoptile from the scutellar notch was observed at the scutellar stage. The coleoptile surface was covered with elongating cells. The coleoptilar stage was characterized by the growth of coleoptile and the development of root. However, aberrant somatic embryos which had leafy scutellum and multiple shoot meristem, were also observed.

Key words : Cryo scanning electron microscopy, Direct somatic embryogenesis, Scanning electron microscopy, Scutellum, Somatic embryo, Surface structure.

Cultured plant tissues undergo two kinds of regeneration pathways. The first is organogenesis and the second somatic embryogenesis. The latter is preferred because of the several advantages, such as for mass propagation, genetic improvement programs, and production of synthetic seeds (Hartmann et al., 1997).

In rice, there are many reports on plant regeneration, either via organogenesis (Abe and Futsuhara, 1986 ; Nakamura and Maeda, 1989 ; Hartke and Lorz, 1989 ; Higuchi and Maeda, 1990) or somatic embryogenesis (Abe and Futsuhara, 1985, 1989 ; Ozawa and Komamine, 1989 ; Jones and Rost, 1989b ; Tsukahara et al., 1996). However, there are conflicting accounts on the regeneration process of rice callus, either via organogenesis or somatic embryogenesis. One of the most convincing somatic embryogenesis seems to be direct embryogenesis on scutellum (Jones and Rost, 1989b).

Somatic embryogenesis is an amazing process because the bipolar structure possessing shoot and root resembling zygotic embryo, is produced from somatic cells. Williams and Maheswaran (1986) reported that somatic embryogenesis could be induced through indirect and direct somatic embryogenesis. Direct somatic embryogenesis is advantageous because there is no intervening callus stage and somaclonal variation can be reduced.

Few morphological studies have been made on the somatic embryogenesis in rice. According to Roberts et al. (1985), the cell surface is the entire assembly of cell wall, plasma membrane and cortical cytoskeleton. The dynamic and integrated assembly as a whole is now considered to play a vital and central role in defining cell shape, cell polarity and morphogenesis in plants. To investigate the regeneration process of rice culture, we examined the process of somatic embryogenesis morphologically, especially, by scanning electron microscopy (SEM).

Materials and Methods

1. Materials

The rice (*Oryza sativa* L. cv. Nipponbare) plants were grown in a greenhouse from May to September 1996. The immature seeds 1 month after anthesis were harvested and kept in a refrigerator before use. Immature caryopses were used as the materials of this experiment.

2. Immature zygotic embryo culture

The immature caryopses were surface sterilized in 70% alcohol for 1 min followed by 1% Na-hypochlorite for 20 min. The caryopses were then rinsed 4 times with

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Abbreviations : Cm, cellulose microfibril ; Cp, coleoptile ; cw, cell wall ; ec, elongate cell ; EN, embryogenic nodule ; f, fibrillar ; GSE, globular somatic embryo ; L, Leaf ; LSc, leafy scutellum ; mls, mesh-like structure ; msm, multiple shoot meristem ; NEN, non-embryogenic nodule ; P, proembryo ; R, root ; S, shoot ; Sc, scutellum ; sm, shoot meristem ; sn, scutellar notch ; t, trichome.

sterilized, distilled water and inoculated on half-strength Murashige and Skoog basal medium. After 4 days of culture, the immature embryos were dissected from germinated caryopses and transferred to embryo-induction medium (EIM) (Table 1). The immature embryos were placed with the abaxial regions in contact with the medium (scutellum region was exposed). Ten immature embryos were cultured in each petri dish. This experiment was carried out in 4 replications each with five petri dishes. After 1 week of culture, the scutellum covered by nodular translucent structures was subcultured every week on embryo-maturation medium (EMM). After 2 or 3 subcultures, depending on the development of somatic embryo, maturing embryos were transferred to embryo-germinating medium (EGM) and cultured for 2 to 4 weeks. Then germinating somatic embryos were transferred to half strength Murashige and Skoog medium containing 0.05 mg L^{-1} NAA solidified with 0.25% gelrite for plantlet hardening. Finally, hardened plantlets with well-developed shoots and roots were transferred to sterilized soil for acclimation, and then moved to the green house. EIM, EMM and EGM (Jones and Rost, 1989b) with a modification on carbon source and gelling agent in EGM, according to Tsukahara and Hirosawa (1992) were used for inducing somatic embryo from scutellum of immature zygotic embryo explant. (Table 1). The cultures for induction, maturation and germination were kept in the dark at 25°C. After the somatic embryos germinated, they were placed in light at 25°C for plant development, hardening and acclimation.

3. Scanning Electron Microscopy

The scutellar tissues, after 4 days on EIM, 1 or 2 weeks on EMM and 1 and 2 weeks on EGM were prefixed with 5% glutaraldehyde in cacodylate buffer pH 7.2 at 4°C for 24 hr. Samples were rinsed in 0.1 M cacodylate buffer 3 times and postfixed in the cacodylate buffer containing 2% osmium tetraoxide at 4°C for 12 h. After being rinsed in 0.1 M cacodylate buffer and distilled water, the samples were successively gradually dehydrated in an alcohol series, immersed in isoamyl acetate for 5 min and dried at the critical point in a Hitachi HCP-1 apparatus using $C0_2$ as the transient fluid . Finally, the samples were coated with gold by an ion sputtering apparatus, EIKO IB-3 and observed by SEM Hitachi S-4000 at 15 kV (Higuchi and Maeda, 1990).

4. Cryo-Scanning Electron Microscopy

The samples were fixed in liquid nitrogen for about 30 seconds. Then the fixed samples were rapidly placed in the sample chamber in SEM equipped with a cryo-stage and observed at 5 kV.

Results

Typical somatic embryogenesis was obtained in the present experiment. The following stages of embryogenesis in zygotic embryos of monocots (Hartmann et al., 1997) were characterized in the scutellum during immature zygotic embryo culture :

1. Proembryo stage
2. Globular stage
3. Scutellar stage
4. Coleoptilar stage

Observations of somatic embryo development by stereomicroscopy (SM) gave us only information concerning the change of their shape but those by SEM observations led us more detailed information concerning their surface changes during development. Table 2 shows some char-

Table 1. The composition of media for inducing somatic embryos from the rice scutellum of immature zygotic embryos.

Media	Basal Media	Hormone	Carbon Source	Gelling Agent
EIM	MS	2 mg L^{-1} 2,4-D	3% sucrose	0.8% agar
EMM	MS	1 mg L^{-1} 2,4-D	3% sucrose	0.8% agar
EGM	MS	—	1% sucrose 3% sorbitol	0.4% Gelrite

Table 2. The development of rice somatic embryos observed by SM and SEM.

Somatic embryo stage	Stereo Microscopy	Scanning Electron Microscopy
Proembryo	Translucent embryogenic nodular structure.	Embryogenic nodular structure has smooth surface.
Transition	The same as above.	Fibrillar material was formed on the surface of the embryogenic nodular structure.
Globular	The same as above.	Mesh-like structure was formed on the surface of the embryogenic nodular structure.
Scutellar	White opaque and more compact. The globular structure changed into scutellum and coleoptile.	Formation of notches was seen on the scutellum structure. The ridge of the mesh-like structure lowered. The cells on the coleoptile surface elongated.
Coleoptilar	Coleoptile grew and the root developed. Scutellum rudimented.	The surface of coleoptile became developed and resembled the leaf epidermis.

acteristics of typical somatic embryos during development observed by SM and SEM.

Observations by SEM showed that the cell walls of scutellum cells , after 4 days on EIM, were smooth. The scutellum cells became swollen during culture so that the cell connections between the neighbouring cells were disrupted (Figs. 1A and 1B). After 1 week on EMM, three kinds of structures, proembryo, non-embryogenic nodule and globular somatic embryo, were seen on the scutellum surface (Fig. 2A). In our observation, proembryos appeared following cell division on the scutellum surface and nodular structures following the growth of the proembryos. There were 2 kinds of nodular structures. The first was embryogenic nodular structures and the second non-embryogenic nodular structures. Each structure has its own characteristic on the cell wall. The surface of proembryo cells was smooth (Fig. 2B). Fibrillar materials were formed on the cell wall surface of both non-embryogenic (Fig. 2C) and embryogenic nodular structures (Fig. 2E). However, the fibrillar material was changed to mesh like structure during the process from embryogenic nodular structure to globular somatic embryo. In contrast, fibrillar material of the non-embryogenic nodular structure gradually disappeared during the change to callus tissues. The non-embryogenic nodular structure did not develop to somatic embryo. High magnification of fibrillar material was shown in Fig. 2D. Presumably, it is cellulose microfibril with granular stuctures. The surface of globular somatic embryos is characterized by the occurrence of mesh-like structure (Figs. 3A and 3B). This means that there is transition phase from proembryo stage to globular stage. It was characterized by the appearance of fibrillar material on the embryogenic nodular structure. When the embryogenic nodular structure underwent the change to globular stage, the cell wall gradually changed into a mesh-like structure, as shown in Figs. 2E and 2F. Figure 2E shows the surface changing gradually from fibrillar to mesh-like structure. The fibrillar material gradually formed dense mat of fibers which finally covered the surface of globular embryo. Figure 2F is the magnification of Fig. 2E showing the border between fibrillar and mesh-like structure. It also shows the expanding cells which encountered one another so that the surface became a little wrinkled due to oppression. Figure 3A shows the globular somatic embryo on which the mesh-like structure has been completely formed. Figure 3B is a magnification of mesh-like structures. The formation of mesh-like structure was confirmed by cryo-SEM as shown in Fig. 3C. Therefore, it seemed that a mesh-like structure layer was induced by wrinkling of walls of cells which were protruding and expanding in growing globular embryo. Consequently, the surface of globular embryo was covered with mesh-like structure of cell wall. During its further development, the cell wall structures gradually changed so that the ridge of mesh-like structure became lower. In the subsequent stage, there was no fibrillar nor mesh-like structure. Therefore, it is suggested that fibrillar material and mesh-like structures are formed only during the transition to globular somatic embryo stage. The globular somatic embryos kept on the EMM for 2 weeks, developed a flattened apical region (Fig. 4).

The next stage, termed the scutellar stage occurred within 1 week on EGM. At this stage, the development of scutellum and its notch was observed (Fig. 5A). After reaching this stage, it was easy to distinguish the somatic embryo from non-embryogenic tissues. The somatic embryo developed a structure from the soft and translucent structure to white opaque, hard and compact, whereas the non embryogenic tissue remained soft. The coleoptile with terminal slit at the tip was also observed in the somatic embryo. The cells on the surface of coleoptile elongated. (Fig. 5B). Within 2 weeks of culture on EGM, as the coleoptile had elongated and the root emerged, the scutellum rudimented. The appearance of coleoptile surface resembled that of the leaf epidermis. This stage is termed the coleoptilar stage. Then leaves grew from the slit at the tip of the coleoptile (Fig. 6).

The abberant somatic embryos observed in the present study appeared as a leafy scutellum with multiple shoot meristem at its base (Fig. 7A). The trichomes commonly occurred on the surface of leafy scutellum (Fig. 7B).

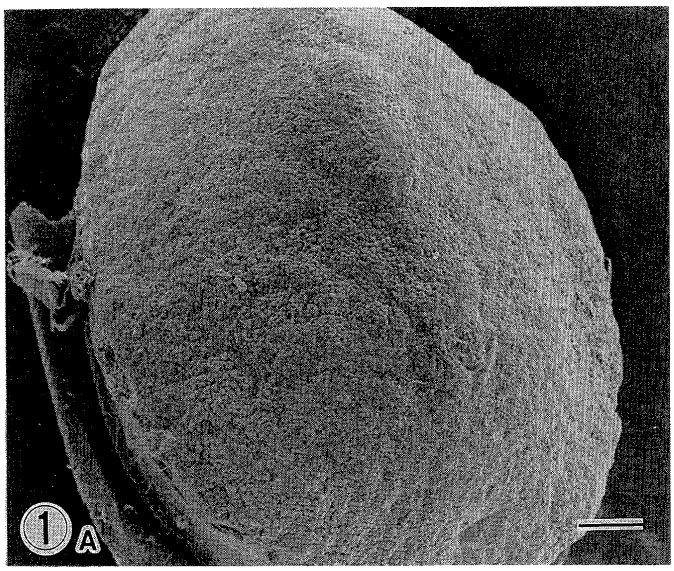
The rate of occurrence somatic embryos which could be converted normally into plantlets was 25% whereas that of aberrant somatic embryos was 17%. The non-embryogenic nodular structure remained in the others, and continued to grow as callus tissue. The germinated somatic embryo was separated from the other tissue and transferred onto hardening media.

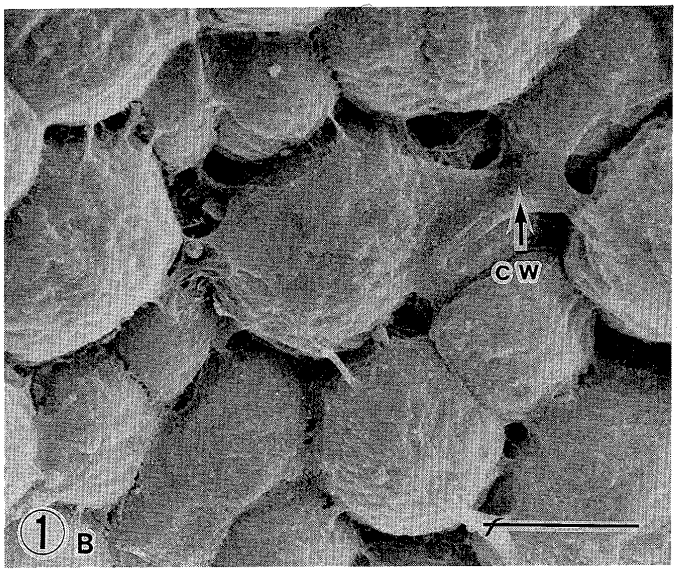
After the roots and shoots were well-developed on the hardening media (Fig. 8), the plantlets were acclimatized on sterilized soil for 2 weeks. Then the acclimatized plantlets were transferred into nonsterile soil in pots and grown in a greenhouse.

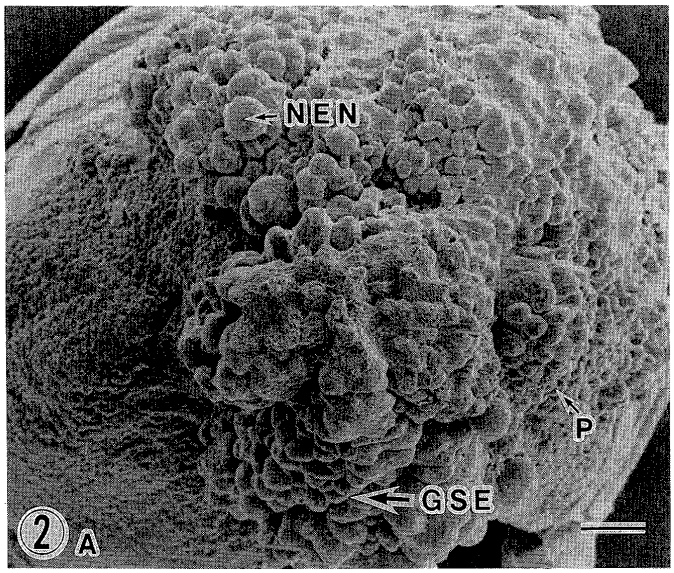
Discussion

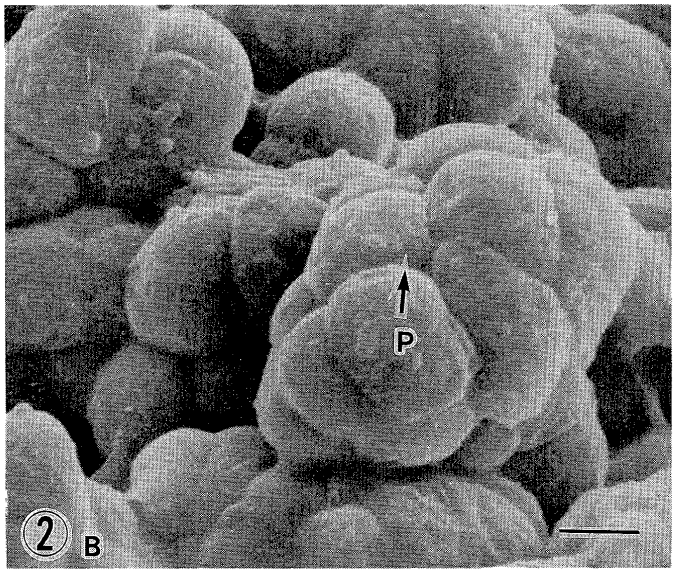
Direct formation of somatic embryos from scutellum epithelial cells has been reported by Jones and Rost (1989b) with various cultivars. They observed it by light and transmission electron microscopy. In the present report, we examined the surface structure during the induction and development of somatic embryos from rice Nipponbare cultivar by SEM.

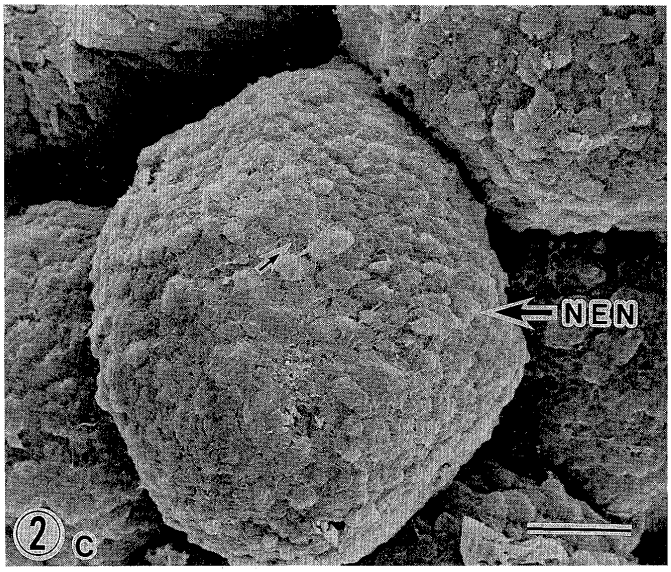
The regeneration capability of cultured rice greatly varies with the cultivar (Abe and Futsuhara, 1986). Nipponbare cultivar has been extensively examined by Nakamura and Maeda (1989) and Higuchi and Maeda (1990). In their studies, plants regenerated through an organogenesis pathway. They used mature caryopses as the explants, but we used immature embryos of immature caryopses as the explants because of the higher response. According to Williams and Maheswaran (1986), immature cells of explants can be induced to

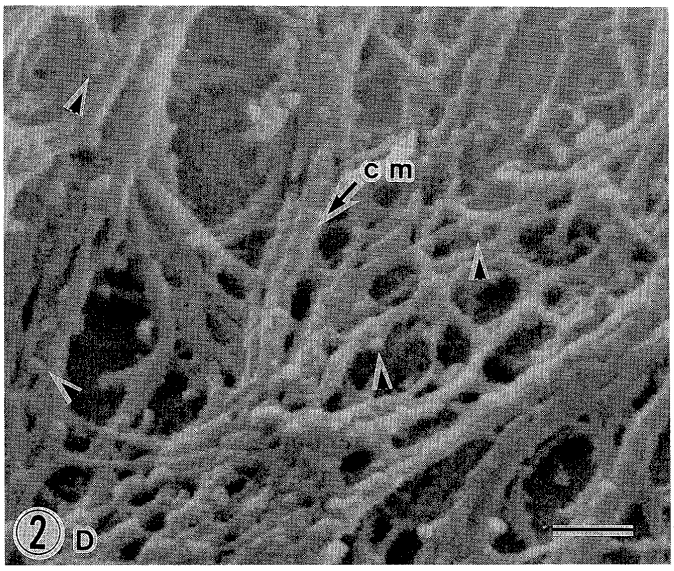






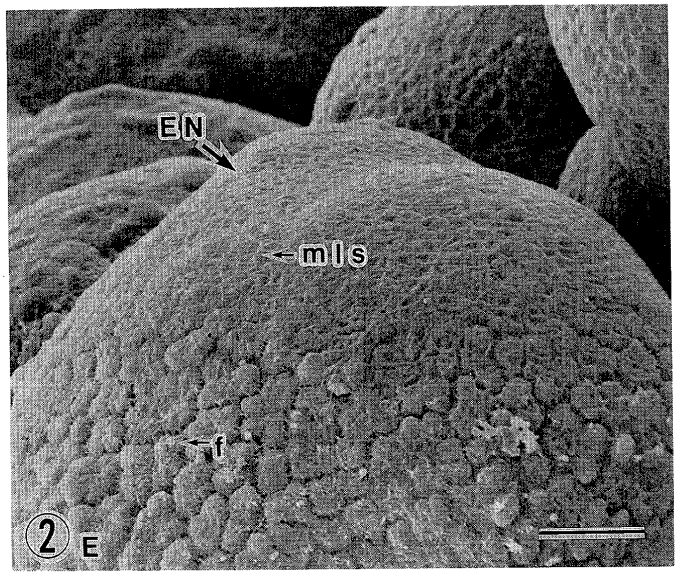


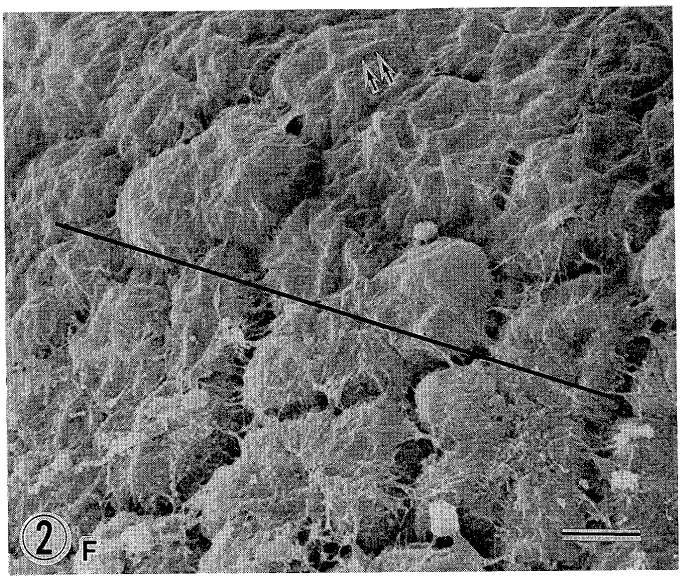


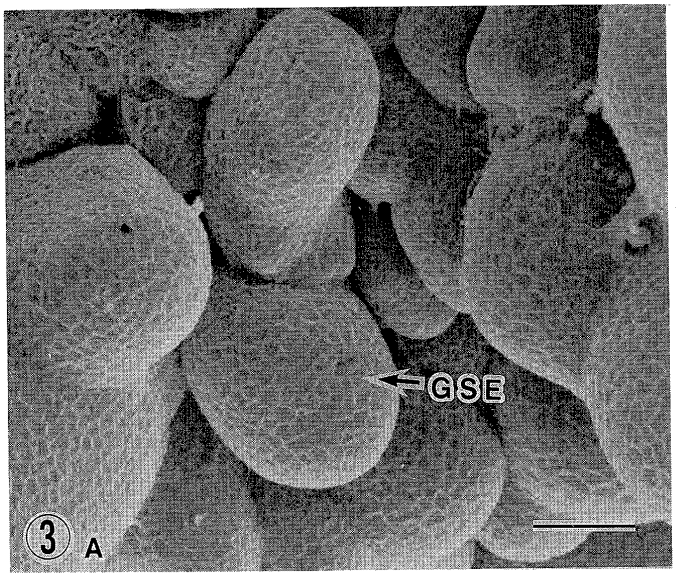


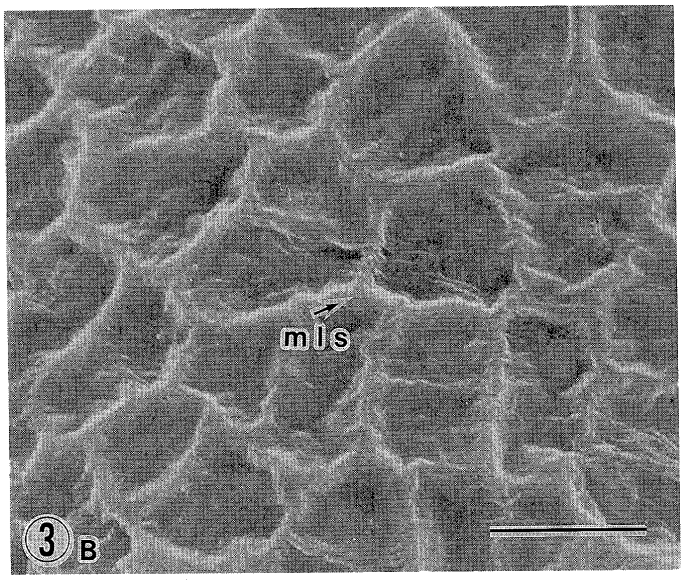
form a somatic embryo through multicellular origin. In mature tissue, only a few immature cells existing among mature cells could be induced to form a somatic embryo and it was characterized by the formation of suspensor-like structure. In this study, somatic embryos may have been induced through multicellular origin because no suspensor-like structure was observed.

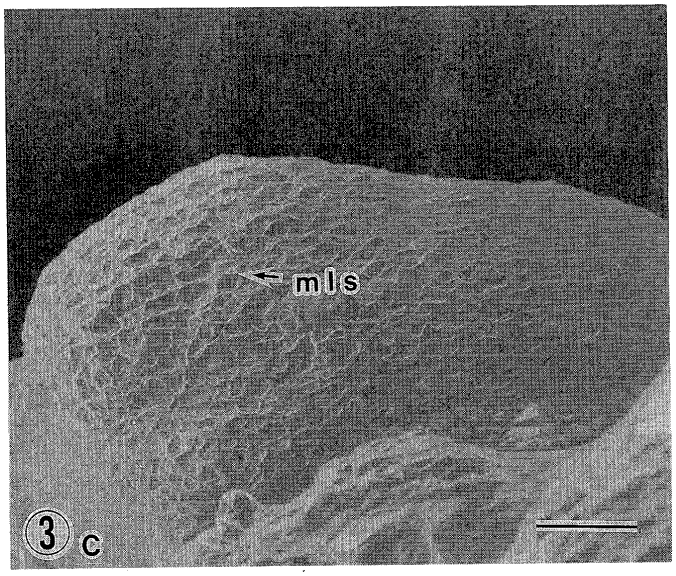
In the scutellum of immature embryos cultured on the medium with a high concentration of 2,4-D, cell connections between the neighbouring cells were disrupted, and

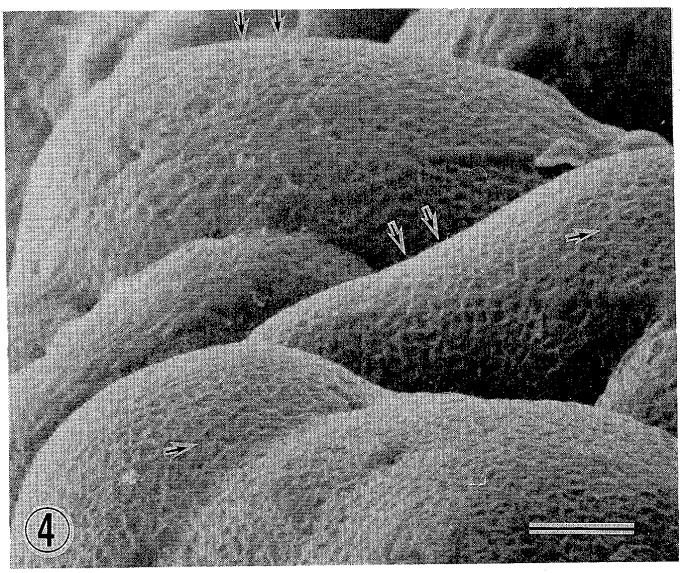




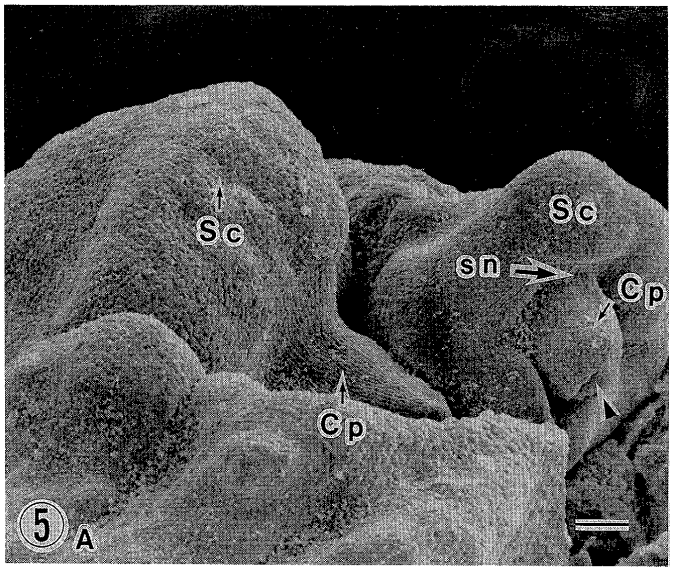


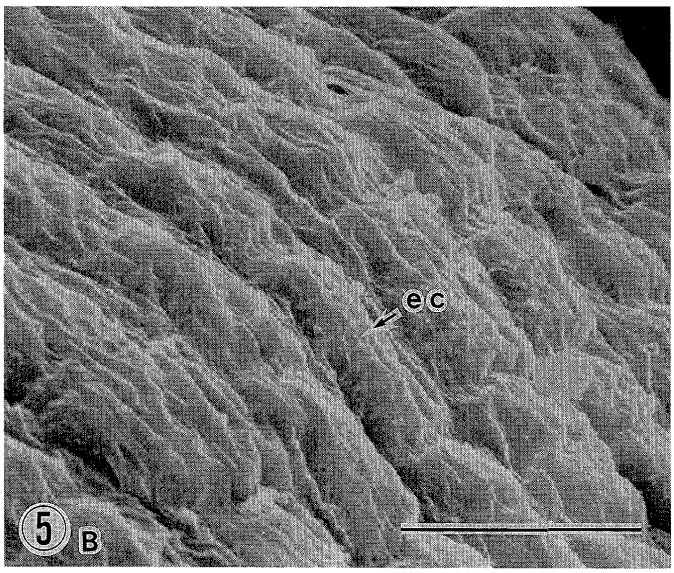


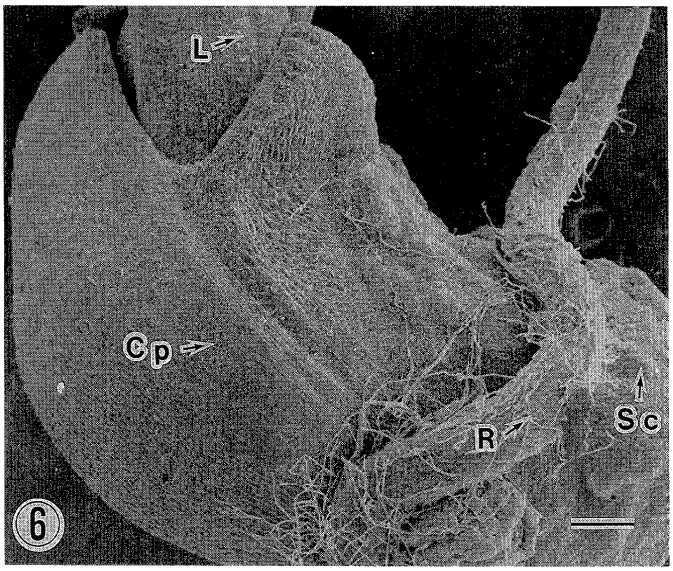


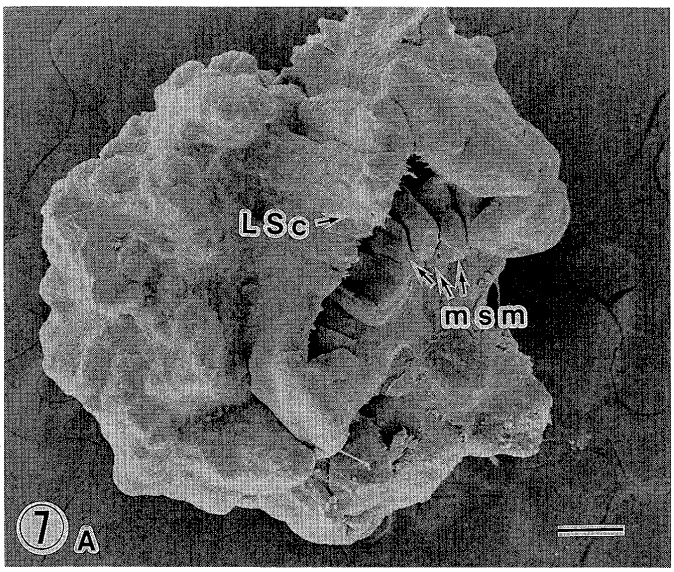


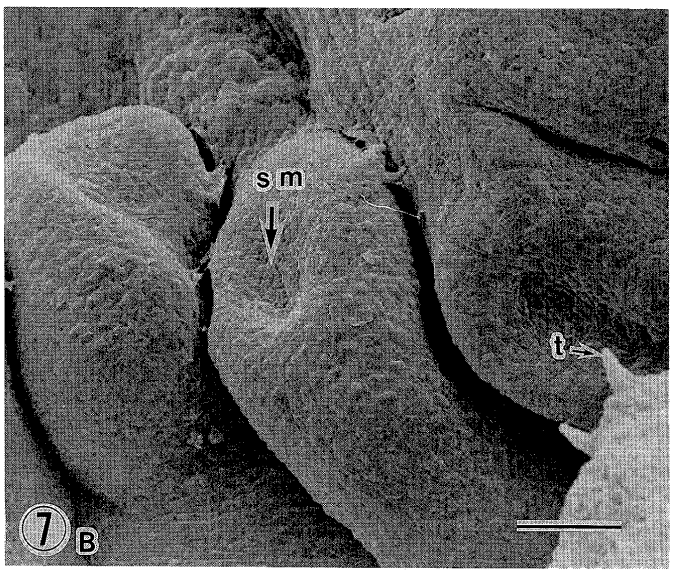
then proembryos and embryogenic nodular structures were produced. Auxin treatment has been suggested to disrupt cell connection before stimulating cell division (Hayashi, 1991). Transfer of the tissue with an embryogenic nodular structure to the medium with a lower concentration of 2,4-D resulted in the transition from proembryo to globular somatic embryo, which was characterized by the occurrence of fibrillar material. This result supports the view of Pedroso and Pais (1995), who stated that the cell wall changes are an in vivo marker of

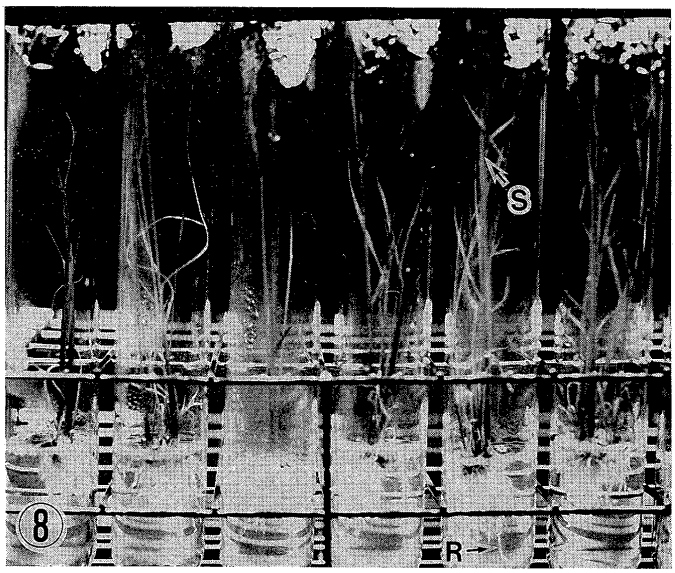












embryogenic competence in the *Cammelia japonica* leaf culture system. They also observed the formation of fibrillar material on the surface of nodular structure which subsequently replaced by the secondary cell wall deposition (cutin). In our study, subsequent morphological change in the cell wall was the appearance of mesh-like structure on the globular somatic embryo. This unique structure distinguishes globular somatic embryos from the nodular structure in the organogenesis. The typical organogenesis of rice callus has been described by Nakamura and Maeda (1989). According to them, the nodular calli transferred to regeneration medium were

Explanations of Figures

Fig. 1. The scutellum of immature zygotic embryo (A) and scutellar cells with smooth surfaced cell wall (B). Note that scutellar cells are characterized by the disruption of cell connections between the neighbouring cells. (A. Bar : 200 μm, B. Bar : 10 μm).

Fig. 2. A. After 1 week of culture on EMM, three kinds of structures are observed. Proembryo, non-embryogenic nodule and globular somatic embryo. (Bar : 200 μm).

B. The smooth surface of proembryo, magnification of proembryos in Fig. 2A. The proembryo consists of 10—12 cells. (Bar : 10 μm).

C. Fibrillar material (arrow) was observed on the surface of non-embryogenic nodular structure. (Bar : 100 μm).

D. Magnification of fibrillar material shows the cellulose microfibril and its granular structure (arrow head). (Bar : 0.2 μm).

E. Embryogenic-nodular structure gradually changing from fibrillar to mesh-like structure. (Bar : 50 μm).

F. Magnification of Fig. 2E. (———) : the border between fibrillar and mesh-like structure. Note the little wrinkled cells surface (double arrow). (Bar : 10 μm).

Fig. 3. Globular somatic embryo covered with a layer of mesh-like structure was completely formed on the surface of (A), and its magnification (B). Confirmation of mesh-like structure by Cryo-SEM (C). (A. Bar : 50 μm, B. Bar : 10 μm, C. Bar : 50 μm).

Fig. 4. The ridge of mesh-like structure became lower (arrow). Apical region of somatic embryos is flat (double arrow) after 2 weeks on EMM. (Bar : 50 μm).

Fig. 5. The scutellar stage characterized by the development of scutellum and scutellar notch. Early stage of coleoptile formed from the scutellar notch. Note the slit at the tip of the coleoptile (arrow head) (A). The surface of coleoptile was characterized by elongated cells (B). (A. Bar : 100 μm, B. Bar : 10 μm).

Fig. 6. Bipolar structure of somatic embryo, consisting of coleoptile and root was observed after 2 weeks on EGM, whereas the scutellum rudimented. The surface of coleoptile resembles the leaf epidermis. The leaf grew from the slit at the tip of coleoptile (Bar : 200 μm).

Fig. 7. The aberrant somatic embryo with leafy scutellum with multiple shoot meristems at its base (A). Trichomes commonly occur on the surface of leafy scutellum. Note the shoot meristem formed (B). (A. Bar : 200 μm, B. Bar : 50 μm).

Fig. 8. The plantlets after 2 weeks on hardening media show the well-developed shoots and roots.

covered with fibrillar material but the surface were smoothed thereafter and then replaced by elongated cells resembling leaf epidermis. Following these processes, leaf primordia was emerged and shoots developed. The above descriptions show that fibrillar material appears in both organogenesis and somatic embryogenesis processes. According to the observations of Pedroso and Pais (1992), the occurrence of fibrillar material was the marker of cells undergoing morphogenesis. Nakashima et al. (1997) considered that the fibrillar material observed during the secondary wall thickenings in cultured cells of *Zinnia elegans* is the cellulose microfibril with granular structure. Such a structure resembles the fibrillar structure in the present experiment. Samaj et al. (1995) stated that the chemical composition and structural arrangement of fibrillar material (extracellular matrix) at the cell surface of embryogenic calli of *Drosera rotundifolia* and *Zea mays* may play fundamental roles in cell recognition, cell to cell interaction, cell division and differentiation, as well as in plant regeneration. Thus, obvious differences exist between the processes of these organogenesis and embryogenesis. In organogenesis, fibrillar material on the nodular structure was replaced by the formation of smooth, elongated cell, whereas in somatic embryogenesis it was gradually replaced by the formation of a mesh-like structure layer, which occurred only on the embryogenic nodular structures. Consequently, it is Williams and Maheswaran (1986) suggested that direct embryogenesis in tissue culture proceeds from the predetermined cells. Non-embryogenic nodular structures which were derived from non pre-embryogenic cells grow into callus tissue. A similar mesh-like structure has been observed on globular somatic embryo of maize (Mc Cain and Hodges, 1986), confirming that the mesh-like structure observed by cryo-scanning electron microscopy was not an artifact of chemical fixation or drying.

Masuda and Tokuji (1996) reported that the irregular cell wall surface in carrot globular embryos was the result of the protrusion and expansion of cells during the development of globular embryo from the proembryo. They presumed that the expanding cells became wrinkled and irregular when they encounter one another. In our experiment with rice, a mesh-like structure was observed on the surface of globular embryo. We suppose that during globular somatic embryo formation, the new cells were protruded and expanded to fill the space between cells, and after encountering neighbouring cells, a wrinkled structure or structure with a ridge and valley (mesh-like structure) was formed on the surface of globular embryos. Furthermore, the protruding and expanding cells are considered to have kept their activity thereafter filling up gaps on the globular to form compact cells, and an epidermal cell layer was formed there in a regular pattern.

The globular somatic embryos subcultured 2 to 3 times on EMM, matured without any obvious morphological change was seen in the appearance of embryos, but with slight elongation and increase in size. When the maturing somatic embryos were transferred to the germination

media, they developed into scutellar stage followed by coleoptilar stage. These processes were similar to those reported on somatic embryogenesis in *Panicum maximum* (Lu and Vasil, 1985). However, immature somatic embryo in the present experiment did not develop to the normal embryo, and formed aberrant embryos instead. The maturation process seems to be very important to obtain the normal somatic embryo development.

Combined use of sorbitol and reduced amount of sucrose seemed to be suitable for rice somatic embryo germination in this study. According to Kishor (1987), the rice callus growing on sucrose as a sole carbon source lost its shoot forming ability by 100 days in culture, whereas callus proliferating on sorbitol and sucrose differentiated shoots over a period of 1500 days. Presumably, these carbohydrates functioned as an osmoregulator besides an energy source. A high percentage of Gelrite in the medium increased the regeneration frequency in cultured rice callus as reported by Tsukahara and Hirosawa (1992). Application of 4 g L^{-1} of Gelrite in the present study caused dessication of the mature somatic embryos and promoted their germination.

The development of somatic embryo without embryo sac resulted in the morphological appearance different from zygotic embryo. According to Suzuki et al. (1996), rice zygotic embryo is about 2.3 mm long 4 days after anthesis. The size of mature somatic embryo was variable, because they grew adjacent with each other, but was 2.5 to 3.5 mm. The somatic embryo seems to be larger than the zygotic embryo because they have no barrier such as the embryo sac.

According to Jones and Rost (1989a), flattening of globular embryos is necessary for scutellum formation in zygotic embryogenesis of rice, just as in somatic embryogenesis reported here. Another similarity between somatic embryo and zygotic embryo is the chronology of organ formation. In zygotic embryo, organ differentiation begins with scutellum emergence at 3 days after fertilization and proceeds basipetally, ending with the delineation of the radicle/coleorhiza by the sixth day. In somatic embryo, the scutellum is also formed earlier than the root. The scutellum formation followed by the growth of coleoptile occurred within 1 week on germination medium, whereas the root developed at the tenth day on the same medium.

The scutellum of the grass embryo is considered to be foliar in organ, and known to develop chlorophyll and leafy structures, and has characteristics of leaf trichomes (Norstog, 1969). The aberrant somatic embryos, for instance the formation of leafy scutellum with multiple shoot meristem were observed in our study. Jones and Rost (1989b) also found the rice aberrant embryos which were often embryos with elongated leaf-like scutella. They assumed that these aberrant somatic embryos occurred due to the precocious germination during late embryogenesis. Wang and Vasil (1982) also observed atypical somatic embryos in the somatic embryogenesis of *Pennisetum purpureum*,, such as green leafy scutellum with characteristic trichomes. In such cases, multiple shoot meristems developed at the base of the leafy scutellum. According to Vasil and Vasil (1982a), in cultured immature embryos of *Pennisetum americanum*, atypical structures are produced instead of typical embryoids. This is probably due to insufficient control in vitro of the endogenous levels of growth substances and the lack in normal physiological gradients and nutrient supply.

In the present study, we examined the somatic embryo derived directly from scutella of immature rice zygotic embryos. The morphology of somatic embryos, such as the cell wall characteristics, formation of notch on the scutellum, development of coleoptile and root was revealed by SEM. Observation of the changes of the cell surface during the somatic embryogenesis in suitable culture conditions by SEM should contributed to the elucidation of the mechanisms involved in somatic embryogenesis.

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