A Scanning Electron Microscope Study on Japonica Type Rice Callus Cultures, with Emphasis on Plantlet Initiation

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Abstract : Morphological details of rice callus cultures were examined at the initial stage of plantlet development, together with the growth of callus established from mature seeds, with the aid of scanning electron microscopy. The observations revealed an occurrence of small knobs on the scutellum region of intact embryos inoculated on a callus induction medium. When transferred to a shoot-forming medium, the callus cultures gradually exhibited sequential changes in the surface structure and finally developed shoots and roots. A mucilaginous membrane layer interestingly covered the surface of globular structure developed on callus cultures. Fibrillar materials interconnected among the cells exposed where this layer was torn. As the callus growth progressed into more advanced stage, the surface structure transformed into a stabilized appearance from fragile nature and enlargement of nodules occurred showing slightly undulated, smooth faced structure. After then, leafy structures emerged on epidermis-like callus surface. They were laminated or cylindrical shapes attending with trichomes and wart-like structures. After these diverse structures appeared, a shoot-like structure finally ensued. And also, apart from the sites of a leafy structure and a young shoot, adventitious roots emerged from the inside of callus masses, and root hairs and root cap-like structures clearly developed.

Key words : Callus, Japonica type rice, Membranous layer, Nodular structure, *Oryza sativa*, Scanning electron microscopy, Shoot-bud formation, Surface stabilization.

日本型イネカルスの走査型電子顕微鏡観察，特に幼植物体形成に関連して：ナカムラ チエミ・前田英三（名古屋大学農学部）

要 旨：日本型イネカルスからの植物体分化初期過程を，走査型電子顕微鏡を用いて研究した．茎葉形成培地上で，カルス塊表面が経時的に変化したのち，不定芽・不定根を生じた．はじめ，カルス塊表面を薄層がおおっていたが，ときに，その層が強く裂かれ，薄層の下から露出した表層細胞に繊維状構造がつかれる．しかし，カルスの生育が進むと，表皮状構造がカルス塊をおおい，より安定化した表面構造を示すようになる．更に塊状構造は肥大・生長し，薄片状または筒状構造を呈するようになり，最終的に幼芽を再生する．また幼芽から離れて，根毛をもった不定根が発生する．従って本研究結果は，カルス表面の安定化が茎葉形成初期過程の一つの特徴であることを示した．

キーワード：イネ，塊状構造，カルス，茎葉形成，走査型電子顕微鏡，日本型イネ，薄膜層，表層の安定化．

Plant tissue culture has been proposed as an excellent technique for the research of plant cell differentiation as well as a supplementary technique for plant breeding programs throughout the use of new and expanded genetic variability. The *in vitro* callus growth and subsequent plantlet regeneration have been already accomplished in numerous species of the dicotyledons and the monocotyledons^{3,12}.

Among the cereals, fortunately, mature embryos and young pollen grains of rice plants are perhaps one of the materials from which the callus cultures have been easily induced to develop plantlets. Substantial information has been already given in reviews on rice callus cultures⁶⁻⁹), but a comprehensive knowledge of the callus growth is not yet present

concerning an initial stage of the developmental process leading to plant regeneration. It is known, however, that the use of scanning electron microscope (SEM) is convenient and effective to examine very precisely the external morphology of various growth and development processes of plants. The ability to observe directly the external features of plant materials with great depth of field and high resolution under a high magnified condition has made the SEM a valuable, excellent tool for the studies of *in vitro* morphogenesis, as well as intact plants *in vivo*.

Accordingly, the present investigation was undertaken for providing the precise figures of surface ultrastructure on the rice callus cultures, especially at the initial stage leading to plantlet regeneration.

Materials and Methods

In the present study, rice (*Oryza sativa* L.) seeds of Japonica type (Aichi-asahi, Aoisora and Aokaze varieties) were used as a source of experimental materials.

For callus induction:

As a callus induction medium, the modified MS basal medium¹⁰⁾ was supplemented with 10^{-5} M 2, 4-dichlorophenoxyacetic acid (2, 4-D)^{8,9,11)}. Each 50 ml of the medium was dispensed in 100 ml flasks and autoclaved at 120°C (1.2 kg/cm²) for 20 min. The seeds were dehusked and surface-sterilized in 70% ethanol for 30 sec and then in 5% sodium hypochlorite for 30 min. And then, they were rinsed three times with sterile distilled water. The sterilized seeds were placed on the

solidified agar surface of the callus induction medium. The callus cultures were maintained at 28°C under continuous light (ca. 3,000 lux) condition. Under the circumstances, callus formation was mainly resulted from the scutellar epithelium of intact embryos. Callus proliferation became visible 7–10 days after seed inoculation.

For plant regeneration:

A shoot-forming medium used for plant regeneration was similar to the callus induction medium except that the concentration of 2, 4-D was decreased to 10^{-7} M and kinetin was newly added at 5×10^{-5} M. The replacement with the shoot-forming medium were made after 4–5 weeks culture on the callus induction medium.

For Scanning Electron Microscopy:

Explanation of figures

Figs. 1 and 2. Surface structure of the callus derived from mature intact embryos cultured on the callus induction medium for 10 days. 1: Callus proliferation from scutellum region, showing several small knob-like structures. 2: Enlargement of small knob-like structure, presuming cell division.

Figs. 3 to 24. Callus cultured for various periods after transfer to the shoot-forming medium.

Figs. 3 to 6. Callus surface after 5-day culture. 3: Membranous layer covering partially the callus surface and fibrillar materials (an arrow) interconnecting among the disclosed cells. 4: High magnification showing membranous layer and fibrillar materials (an arrow). 5: A bundle (an arrow) of fibrillar materials formed on the callus surface. 6: The details of fibril bundle under high magnification.

Figs. 7 to 9. Callus surface after 7-day culture. 7: Various type of nodules on a callus culture. 8: Slightly undulated, smooth surface of a folding broad nodule. 9: A slightly elongated nodule.

Figs. 10 to 12. Fragments of callus cultured for 10 days. 10: Slightly elongated cells arranged at one direction, resembling the surface of leaf epidermis. 11: Protuberances (arrows) from epidermis-like surface. 12: Cylindrical shape of leaf-like structures with or without trichomes.

Figs. 13 to 16. Callus surface after 15-day culture. 13: Leaf-like structure showing laminated and cylindrical portions. Note the both having some trichomes. 14: Root development and laminated leaf-like structure, the latter having both trichomes and wart-like appendixes. 15: Initial emergence of trichomes (arrows) on the smooth surface nodule. 16: Numerous trichomes developed on the epidermis-like surface.

Figs. 17 and 18. Leaf primordium on callus surface after 25-day culture. 17: Young leaf primordia exposed by hand-fracture of the surrounding leaf. 18: The high magnification showing the elongated cells arranged at one direction on the surface of two leaf primordia.

Figs. 19 to 21. Root emergence from callus after 7-day culture. 19: An aspect of root emergence. Note the occurrence of cell elongation. 20: Initial emergence of several roots from a part of callus. 21: Root apex-like structure.

Figs. 22 to 24. Root development after 20 days culture. 22: Development of a root along with root hairs in the vicinity of nodules and a leaf-like structure. 23: The details of secondary root formation. 24: Root hairs under high magnification.

Bars are: 10 μ m in Fig. 6; 50 μ m in Figs. 2, 4 and 24; 100 μ m in Figs. 1, 3, 5, 7, 8, 9, 10, 11, 14, 15, 16, 18, 19, 22 and 23; and 500 μ m in Figs. 12, 13, 20 and 21.

Abbreviations

C: cylindrical leaf structure, H: hand-fractured leaf, L: laminated leaf structure, M: membranous layer, N: nodular structure, R: root, S: smooth surface, SR: secondary root, T: tip of root.

Callus fragments at various growth stages were removed from the culture flasks, transferred to a fixative solution of 3% glutaraldehyde and 1.5% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.2) and fixed for 5 hours shaking gently at room temperature. The fixed tissues were rinsed for 10 hours with two changes of 0.2 M cacodylate buffer at 4°C. After dehydration through a graded ethanol and isoamyl acetate series, the specimens were critical point-dried in a Hitachi HCP-1 apparatus with CO₂ as the transition fluid. The dried samples were mounted on metal stubs with double-stick tape, fixed with electroconductive silver paste and gold-coated (7 mA) for 7 min using an Eiko IB-3 ion coater. The specimens were examined on a Hitachi S-415 scanning electron microscope operating at 25 kV and the photographs were taken.

SEM micrographs of Aichi-asahi var. callus cultures are shown in all figures, except Figs. 15 and 16 of Aoisora var. and Figs. 20 and 21 of Aokaze var. There were no essential differences in the varieties used.

Experimental Results

Macroscopic observations with the naked eye were able to find a definite swelling on the scutellum region of mature rice embryos after 7 days culture on the callus induction medium. Developmental sequences of the callus growth were examined by SEM level. After 10 days culture, the callus masses occurred overall on the scutellum region of embryos and they consisted of several small knob-like structures (Fig. 1). An average diameter of the small knob-like structure was about 25 μ m, assuming the size of each superficial cell in the callus cultures. Cell division in the small knob was imaginable from the surface ultrastructure as shown in Fig. 2 which is indicative of new cell wall orientation probably giving rise to four-cell groups (tetrads) in a small knob.

To examine the developmental processes of callus cultures on the shoot-forming medium, the materials were sequentially sampled 5, 7, 10, 15, 20 and 25 days after transfer into the shoot-forming medium. Several different structures were macroscopically detected on the callus surface after 5-day culture. At 7 days, the initial green spots along with small leafy outgrowths occurred only on the callus

surface in closely contact with the medium.

By SEM level, the globular or rounded structures were found on the growing callus mass surface at 5 days of culture, interestingly they were almost covered with slime-like membranous materials (Figs. 3 and 4). However, the membrane covering was ruptured at some places, and fibrillar materials resulted from the break down of the membrane layer. These fibrils interconnected among the superficial cells which exposed from underneath this broken layer. Sometimes, bundles of fibrous structure were ensued from this membrane rupture, as shown in Figs. 5 and 6. These features were suggestive of a fragile nature of membranous materials covered the callus surface. Some slime-like membranous layer was still present on the surface of large nodules after 7 days of culture (Fig. 7). However, sometimes, the nodules were covered with smooth or rough surface. The rough surface was often on the small nodules, while the smooth surface was slightly undulated showing an unfragile appearance over the developed nodules and it was composed of rather isodiametric cells. Furthermore, broad nodules were formed with smooth appearance in the surface (Fig. 8) and in another case, the nodules were slightly elongated (Fig. 9).

When cultured for more than 10 days, the cells located on the callus surface were slightly elongated and arranged with a single orientation (Fig. 10). This was indicative of the initial regeneration of epidermis-like layer, which involved the transformation of callus surface into more stabilized nature from the fragile property. Emergence of small protuberance from the epidermal layer was seen (Fig. 11). At 15 days of culture, the nodules were more elongated and grew as leaf-like outgrowths with or without trichomes on leafy epidermis (Fig. 12). At more advanced stage, these outgrowths were cylindrical or laminated shapes and had more trichomes (Figs. 13). In the vicinity of these structure, an adventitious root developed as seen in Fig. 14. In Figs. 15 and 16, it is observable that trichome initials and prickle-shaped trichomes emerge on the epidermis-like surface which has probably an epicuticular nature.

In the callus cultures after 25 days, two leaf primordia were disclosed by hand-fracture of the surrounding leaf as shown in Figs. 17 and

18. They were located at the opposite site to each other, assuming the differentiation of a shoot apical meristem enveloped by them.

The root tips were emerged from the inside of callus mass after about 7 days of culture. Several of the root tips arose as rough surfaced clusters from a restricted area of the callus mass (Figs. 19 and 20). According to the growth of roots, longitudinally arranged slimy pieces were beginning to peel off along the slender cells of root tips (Fig. 20). Sometimes slimy materials were observable over a root tip (Fig. 21). After 20 days (Fig. 22), the development of root hairs was displayed together with a leaf-like structure and a nodular structure. A secondary root diverged from the primary root with root hairs as seen in Fig. 23. A high magnification clearly revealed the root hair development (Fig. 24). Exhibiting the above mentioned sequential events, rice callus cultures grew to a bulky mass and developed into a complicated structure. Furthermore, the plantlets derived from these callus cultures exhibited the relatively normal morphology.

Discussion

At present, successful aseptic culture methods permit organ formation in callus cultures derived from numerous plant species. In this paper, mainly, we attempted to clarify ultrastructure of callus surface leading to an initial stage of plantlet regeneration. The mature rice seeds of Japonica type were used to obtain callus masses. The formation of initial callus was examined at scutellum region of intact rice embryos on the callus induction medium.

Growth and development of callus cultures derived from rice seeds as well as other monocot plant callus cultures are more remarkably complex in the external morphology than dicot callus cultures, in addition to the histological difference⁷⁾. The initial stage of differentiation into plantlets is more complicated to be analysed than the callus growth¹¹⁾. Especially, determination of somatic embryos in monocot plants is very difficult, because the pattern of zygotic embryogenesis itself is very diverged in accordance with the used plant species. Consequently, the ultrastructural study by SEM of the callus cultures in monocot plants is fragmentary, especially in Gramineae.

Several morphological traits of callus surface have been described using Gramineae callus cultures: (1) the elongated circular leaves and shoot primordia on the callus cultures derived from the nodal region of maize embryos¹⁴⁾, (2) the compact nodular structures and leafy structures on the callus cultures derived from the scutellar cells of immature embryos of *Sorghum bicolor*²⁾ and (3) organization of both disc-shaped scutellum and cup-shaped scutellum enclosing the coleoptile on the callus cultures derived from young leaves of *Pennisetum purpureum* Schum⁴⁾.

Callus induction from intact rice embryos and subsequent plant regeneration have been revealed by several papers, but the ultrastructural details are not yet sufficient concerning the initial stage in developmental process of callus cultures, leading to the formation of shoot-buds and adventitious roots.

Inoue and Maeda (1980), utilizing the SEM in rice callus studies, indicated about 100 to 500 μm in the diameter of the nodule-like masses on which fine globules of about 10 to 30 μm in the diameter were formed. Our research found the small knobs, each was of about 25 μm diameter, assumed the size of each superficial cell. To closely analyze the superficial traits of callus cultures, however, it is necessary to ascertain histologically whether the diameter of the cells constructing small knobs coincides with that of the surface cells observed by SEM or not.

The callus cultures are composed of globular structures which partially coated with sheet-like layer. These globules are more or less round. The surface layer is a smooth appearance and fibrous threads are formed at the margin where the layer was torn. The fragile nature of the external materials coated on the superficial cells is apparent on most of the callus at the initial stage. The slime-like membranous materials, sometimes, are tore to form bundles of fibrous structure. Surface stabilization of the callus is essential for shoot-bud formation. In the shoot-forming callus, a stabilized epicuticular layer seems to develop gradually on the outer surface of the superficial cells. Interestingly, the breakages of membranous covering on surface cells and extrusion of newly formed cells have been found in sugarcane explants inoculated in the culture medium¹³⁾.

The differentiated regions on the more aged callus possess many variable protuberances. Maeda et al^{8,9)} utilized the SEM to display the smooth callus surface, nodule structure, tubular outgrowth, wart-like structure and trichome on callus cultures derived from rice seeds. Epidermis-like cell layer and trichome were already exhibited on the callus cultures. Abe and Futsuhara¹⁾ utilized the SEM to show the differentiating shoots from the callus cultures derived from rice seeds.

In our study, the highly magnified view of leaf primordial structure can be seen in Fig. 18. It is expected from the figure that a shoot apex is enclosed by these leaf primordia. Several research reports have indicated the occurrence of coleoptile-like or scutellum-like structures on *in vitro* callus cultures. Consequently, a comparative study of plant tissues cultured *in vitro* with that developed *in vivo* is very important for an exact identification of these structures induced by *in vitro* culture. A detailed comparison, however, is not possible without an achievement of comprehensive studies at the spatial and temporal aspects of the architecture of callus cultures.

To obtain high frequency and desirable processes of plant regeneration through rice tissue culture is indispensable to refine more exactly ultrastructural knowledges on external and internal features of the callus cultures at the initial stage of both shoot-bud and root formation.

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