Optical Microscopy and Scanning Electron Microscopy on the Surface of Rice Callus after Treatment with Cell Wall Degrading Enzymes

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Abstract: Two distinctive structures were observed on the surface of rice callus treated with cell wall degrading enzymes, pectinase and cellulase by scanning electron microscopy; i.e., membranous, and the fibrillar structures. The membranous layer was digestible with the enzymes, while the fibrillar structures were not. The membranous matrix was mucilaginous, and positive to PAS reaction. Protoplasts were released after the enzyme treatment from the areas covered with the enzyme-digestible mucilaginous layer. The role of the extracellular matrix of the rice callus is discussed from the standpoint of growth and differentiation of the rice callus.

Key words: Enzyme digestion, Mucilaginous membrane, Periodic acid-Schiff (PAS) reaction, Protoplast, Rice callus, Scanning electron microscopy.

The growth and differentiation of cultured cells, tissues and calli, which originate from rice (Oryza sativa L.) seeds and seedlings, have been studied extensively (Raina, 1989; Christon, 1994). However few morphological studies on the growth of rice calli have been carried out (Maeda 1967, 2000; Inoue and Maeda, 1976; Maeda et al., 1986). In particular, topographical details of the callus surface have not been studied, except for the studies by Nakamura and Maeda (1989) and Higuchi and Maeda (1990). They found using SEM that fragile slime-like membranes, rigid fibrous structures, and thin layers of epicuticular nature were discernible on rice callus.

The surface of the plant is generally covered with the water-impermeable outer wall of epidermal cells, and protoplasts are hardly obtained from the epidermal surface. Therefore, rice protoplasts have been successfully isolated from the chopped sections of roots and leaves and not from the epidermal layer (Maeda, 1971; Maeda and Hagiwara, 1974). Sato et al. (1995a) isolated numerous protoplasts from the chopped samples of petunia leaves and rice calli, and further reported a practical fixation method of isolated protoplasts for SEM.

The outer surface of the rice callus usually is not covered with rigid walls, and the whole surface of the growing callus are mostly covered with mucilaginous matrix moistened with the culture medium used (Maeda, 2000). The treatment with cell wall degrading enzymes may digest the mucilaginous wall matrix on the cell surface, and allow the release of rice protoplasts from the callus masses.

Even though the coat matrix of callus surface influences the cell-wall digestion for the isolation of protoplasts from the callus surfaces, the surface architecture of the rice callus has not been examined yet. Therefore, we examined the coat matrix of rice calli treated with cell wall degrading enzymes with optical and scanning electron microscopes to clarify the nature of surface stratum of the calli.

Materials and Methods

1. Plant material

Rice callus was induced from mature seeds of Oryza sativa L. (cv. Nipponbare). After dehusking, the seeds were exposed to 70% ethanol (v/v) for 30 sec to remove fatty substances and then surface-sterilized with 5% sodium hypochlorite (v/v) for 25 min. The sterilized seeds were washed three times with sterilized-distilled water and placed in 100 mL flasks containing 50 mL of modified MS basal medium (Murashige and Skoog, 1962) supplemented with 10^{-6}M 2,4-dichlorophenoxyacetic acid (2,4-D), 78.4 mg L^{-1} Fe-EDTA, 200 mg L^{-1} myo-inositol, 1.0 mg L^{-1} thiamin HCl, 3 g L^{-1} casein hydrolysate, 30 g L^{-1} sucrose and 6.5 g L^{-1} INN-agar (BA-10). The pH was adjusted to 5.8 before autoclaving. The initial cultures were derived mainly from scutellum in the intact seed embryos and maintained at 25°C under continuous light (40.5 μmol m^{-2} s^{-1}). After 30 days, calli were subcultured on the same medium and they were used for experiments within one month of subculture.

2. Treatment with wall-degrading enzymes

The whole callus mass, about 2.5 mm in diameter, was


Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; PAS, periodic acid-Schiff; SEM, Scanning electron microscopy; TB, toluidine blue.
carefully removed from the culture flasks while retaining its shape, and five callus blocks were incubated without shaking for various periods in 2 mL of an enzyme solution containing 1% Macerozyme R-10 (w/v) (Yakult Honska, Japan), 4% Cellulase Onozuka RS (w/v) (Yakult Honska, Japan), 10 mM CaCl₂•2H₂O and 0.4 M mannitol (pH 5.8). After the incubation, the callus blocks were rinsed in solution A containing 0.4 M mannitol and 10 mM CaCl₂•2H₂O (pH 5.8). The prepared specimens were observed with SEM and histobiochemical procedures.

3. Scanning electron microscopy
The materials were fixed overnight in a mixed solution of 2% glutaraldehyde and 1% tannic acid in solution A at 4°C, rinsed in solution A and post-fixed in 1% aqueous osmium tetroxide for one hour at room temperature. The specimens were dehydrated using a graded ethanol series at intervals of 20 min. Final drying was done using isomyl acetate and carbon dioxide. Dehydrated specimens were covered with gold under vacuum conditions and then examined with a Hitachi scanning electron microscope (S-415) operating at 25 kV. Details of these methods have been described elsewhere (Sato et al., 1995a).

4. Optical microscopy
The materials were fixed in formalin-acetic acid-70% alcohol solution (O’Brien and McCully, 1981) for 20 h, then rinsed three times in 0.1 M cacodylate buffer (pH 7.4) at 4°C before dehydration with a graded ethanol series. They were then embedded in methacryl resin (glycol methacrylate: Quetol 525: 85: 15 v/v, Nisshin EM Co., Japan), cut into 2 μm thick sections using a Reichert-Nissei Ultracut N ultramicrotome, and stained with periodic acid-Schiff (PAS) or toluidine blue (TB) solution (O’Brien and McCully, 1981) prior to microscopic examination.

Results
The whole surface of the growing rice callus was smooth and undulated (Fig. 1). Small bulges were seen in places on the surface.

After a 1-h incubation with the enzymes, the extracellular membranous matrix of the callus surface was selectively digested (Fig. 2). This showed that the membranous matrix was composed of enzyme-digestible mucous material. The surface stratum of cali was usually exposed by the removal of outer wall matrix and many protoplasts were released from the cells in the digested areas (Fig. 3).

Large and undigested cali showed different surface morphology (Fig. 4). On the callus, many fibrils connecting the ruptured membranous matrix layer were observed. The membranous layer appeared to be stretched over and split off where the fibrils were visible. The rupture was probably caused by the increased callus mass in volume (Maeda, 2000).

By the enzyme treatment, protoplasts were released from both the digested sites of the membranous matrix and the sites around undigested fibrils (Figs. 5 and 6). The fibrils were observed after the removal of the mucous matrix layer. Even after a 4-h incubation in the enzymes, fibrils remained persistently on the callus surfaces. Many protoplasts were released from the sites where the fibrils were not located.

The surface of the rice callus was covered with PAS-reactive polysaccharides (Figs. 7 and 8). After a 1-h enzyme treatment, the PAS-reactive polysaccharides were partly digested and removed (Fig. 9). Two hours after the start of the enzyme treatment, much more polysaccharides were removed, and the cell walls were

Explanation of figures
Fig. 1. Rice callus before the treatment with cell-wall degrading enzymes, showing the membranous layer on the callus surface (bar : 60 μm).
Fig. 2. The surface of the callus treated with the enzymes for 1 h. Note the partial digestion of membranous layer (m) (bar : 60 μm).
Fig. 3. High magnification of the enzyme-treated callus showing a number of released protoplasts (p) on the callus surface 1 h, after the start of the enzyme treatment (bar : 20 μm).
Fig. 4. The rice callus surface before the treatment with enzymes, showing fibrils (arrows) interconnecting the ruptured membranous layer (bar : 60 μm).
Fig. 5. The surface of the callus treated with enzymes for 1 h. Note the presence of undigested fibrillar structure and the release of protoplasts (p) (bar : 60 μm).
Fig. 6. High magnification of enzyme-treated callus showing protoplasts appeared between fibrillar structures (bar : 20 μm).
Figs. 7 and 8. Sections of rice callus before the treatment with the enzymes showing the surface covered with PAS-positive polysaccharides (arrows) (bar : Fig. 7, 50 μm; Fig. 8, 20 μm).
Fig. 9. A PAS-stained section of rice callus treated with the enzymes for 1 h. Note the digestion of covered polysaccharide (arrow) (bar : 50 μm).
Fig. 10. A TB-stained section of rice callus treated with the enzymes for 2 h. Note the absence of polysaccharides and the presence of separated protoplasts (p) (bar : 50 μm).

Abbreviations on figures: m, membranous layer; p, protoplast.
also partially digested. Many gaps appeared among the callus cells, and finally the protoplasts were released from the callus (Fig. 10).

Discussion

Land plants need to conserve water in their tissues under a dry air environment. The inner tissue of the plants is covered with epidermal cells, which are coated with water-impermeable fatty cuticles. However, some parts of the plant such as root caps have a mucilaginous coating mainly consisting of both glycoproteins and polysaccharides. Polysaccharide slimes in the maize root caps have been studied in detail (Paull and Jones, 1976). Iijima and Kono (1992) stated that mucilage was visible at the outermost layers of the cap cells in maize roots. It has been suggested that mucilage of rice callus may be secreted from the root tips that emerged on the calli cultured on regeneration media (Nakamura and Maeda, 1989).

At the early developmental stage of carrot embryos, Lackie and Yeung (1995) found that cuticular materials were deposited on the outer tangential wall of protoderm cells but not on the cell walls of suspensors. They described that the protoderm was compatible with a primary meristem from which the epidermis with a cuticular layer differentiated (Lackie and Yeung, 1995). The outer surfaces of the growing rice calli were covered with mucilaginous matrix, and a shoot meristem arose from the epicuticular layers regenerated on callus surface in a similar way as the zygotic embryos of rice (Maeda, 2000).

External morphology of rice callus has been studied in relation to adventitious shoot formation (Maeda et al., 1986; Nakamura and Maeda, 1989; Higuchi and Maeda, 1990, 1991). Two different types of surface architecture were found in their studies, friable and compact calli. On solid media, shoot formation was frequently observed in the globular compact calli spotted with small white-colored regions. They were dry and firm in appearance and partially covered with cuticular layers. The vigorously growing friable calli with yellow wettish surfaces developed shoots at a low frequency.

It was also reported that plantlets of Viola patrinii were often regenerated from green compact calli but not from friable calli with a light-brown colored surface (Sato et al., 1995b).

Gas chromatographic analysis of mucilage extracted from soybean calli indicated the presence of glucose and galactose (Keese et al., 1991). Honda et al. (1997) showed that glucuronomannan polysaccharides, major components of extracellular materials, were deposited in the intercellular spaces of the surface cells in tuberose callus mass. Furthermore, the presence of arabinogalactan-proteins with high water-holding capacity was revealed on the outermost cell walls of embryogenic cells in maize calli (Šamaj et. al., 1999a) and the localization was demonstrated by an immunogold SEM technique (Šamaj et. al., 1999b).
Dubois et al. (1992) found a fibrillar network linking the surface cells of *Cichorium* cali, and reported that the network was digested with protease. Šamaj et al. (1995) reported that a highly developed extracellular matrix was located on the surfaces of the callus in sundew and maize. They described that proteinaceous substances were involved in the matrix, and the bridges with net-like materials were inserted between the cells. During the development of somatic rice embryos from immature caryopses, fibrillar materials which were probably compatible with cellulose microfibrils, were observed at the transition period from proembryo stage to globular stage (Mariani et al., 1998).

When plant tissues enveloped in the epidermis were incubated in cell wall degrading enzymes, the epidermis cells were hardly digested because the epidermis is covered with chemically stable epicuticular materials such as cutin and the related substances.

The fibrils often appeared in the mucilaginous matrix on the rice callus as the callus enlarged, and the timing of the appearance of fibrils corresponded with that of white-colored regions on the compact callus. It is most likely that such fibrils consisted of the substances stable to pectinase and cellulase action. The extracellular matrix surrounding the cells and tissues of rice callus was constructed with the surface stratum such as mucilaginous, fibrillar and epicuticular layers. The nature of the extracellular matrix may be reflected in the responses to the wall-degrading enzymes. It may play a significant role in the determination of cell growth at the callus surface and shoot differentiation.

In the present study, the membranous matrix and the fibrillar structure were separately observed on the surface of the rice callus by SEM. The membranous layer was digestible with a combination of pectinase and cellulase, but the fibrils were not.

We conclude that the outermost surface stratum of the rice callus mainly consisted of the membranous layer and fibrillar structure, which was revealed by SEM after the treatment with wall-degrading enzymes. The mucilaginous membrane was identified as a PAS-positive extracellular matrix. The matrix was susceptible to the digesting enzymes, while fibrils were resistant to the enzyme action. Spatial and temporal changes in the surface stratum in the rice callus are probably interesting objects for examining the mechanism of shoot regeneration on the rice callus.

References


* In Japanese.
** In Japanese with English summary.