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Microtopography and Shoot-bud Formation of Rice (*Oryza sativa*) Callus

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Abstract

The scutellar epithelium of rice seeds shows a variety of activities under different physiological conditions. The epithelial cells cease activity soon after the start of germination, but in the seminal root culture system, they retain their haustorial property for a long period. Furthermore, on solid medium with added 2,4-D, they divide to form callus masses that lack a cuticular layer. The two-step method of callus culture results in a higher frequency of shoot-bud formation that is related to topographical variation on the callus surface. The development of a compact callus with white and green patches is a morphological prerequisite for shoot-bud formation. The cells constituting a callus mass behave differently according to the positional information determined by the internal and external environment. The external appearance of the callus largely depends on the extracellular matrix. The callus surface consists of a fragile slime-like membrane, firm fibrils, and a thin epicuticular layer. Finally, it is suggested that the newly formed cuticular layer on the callus surface is essential for shoot-bud formation. Consequently, we propose that the topographical characteristics of the callus mass play an important role in shoot-bud formation.

Keywords: abscisic acid, callus surface, extracellular matrix, *Oryza sativa*, scutellar epithelium, shoot apical meristem, topographical variation, water stress,white patch, zygotic embryogenesis.

Introduction

The cytohistological details of *in vitro* organogenesis in plants play an importance role in shoot formation on callus cultures. To date, only limited success in this field has been obtained with cereal plants. The growth and differentiation of callus cultures produced from the seeds and seedlings of rice plants have been studied extensively at the morphological level (Raina, 1989; Christon, 1994). However, few cytological studies of the growth of rice callus have been carried out. In particular, little is known about the topographical details of the callus surface. Since 1965, we have studied the differentiation of shoot apical meristem on rice callus. The external morphology of rice callus has been clarified in relation to shoot-bud formation (Maeda *et al.*, 1986; Nakamura and Maeda, 1989; Higuchi and Maeda, 1990). Two different approaches are used to study shoot-bud formation on the callus mass. One uses various combinations of physical and chemical factors to induce shoot buds and the other traces the morphological events leading to bud formation. The former needs well-controlled, large installations, and the latter involves a careful study of callus surface. This review concentrates on the latter approach.

Multi-functional scutellar epithelium

Immediately after fertilization, the rice (*Oryza sativa* L.) egg cell is covered by a thin plasma membrane (Maeda and Maeda, 1990). The cuticular layer is absent from the surface of globular embryos until 66 h after anthesis, as shown by the absence of materials that react with iodine/potassium iodide-sulfuric acid-silver proteinate (the silver proteinate method) (Suzuki *et al.*, 1993). Using the silver proteinate method, the cuticular layer is initially detected on the ventral surface of the embryo 3 days after anthesis; it then spreads rapidly to cover the

entire surface of the embryo. Consequently, epoxides are evident on the surface of young embryos, and it has been suggested that cutin and the related substances constitute the electron-dense layer (Wattendorff, 1974; Wattendorff and Holloway, 1980).

Four days after anthesis, the surface of the embryo is completely surrounded by a thin electron-dense layer. Transmission electron microscopy (TEM) shows that this layer is continuous, even on the dorsal surface (**Fig. 2**). However, this layer begins to disappear from many locations on the dorsal side of embryo 5 days after anthesis (**Fig. 3**). Although the silver proteinate method detects deposits of silver granules as a thick, continuous layer on the ventral surface (**Fig. 4**), the layer becomes discontinuous on the dorsal side (**Fig. 5**) and is completely absent from the dorsal surface 6 days after anthesis. Consequently, scutellar epithelial cells form on the dorsal surface of the embryo (Suzuki *et al.*, 1991). By removing the cuticle, the epithelial cells develop a haustorial function, allowing them to absorb endosperm material during embryogenesis and germination. The first sign of the shoot apical meristem on the upper part of the ventral side in the globular embryo occurs 3.5 days after anthesis (Suzuki *et al.*, 1994). From a face view, the apical meristem consists of a 60x80-μm almost circular area, about 10 protodermal cells across vertically by 13 protodermal cells horizontally. Consequently, the ventral surface is a place peculiarly suited for the first appearance of the cuticular layer and for the shoot apical meristem to arise.

These findings indicate that there is a spatial relationship between formation of the shoot apical meristem and the early appearance of the cuticular layer within a limited area. In summary, there is dorsiventrality in the development of rice embryos. On the ventral side, the epicuticular layer is initiated early on and the shoot apical meristem is organized immediately afterwards. On the dorsal side, the layer soon disappears and the epithelial cells are capable of dividing vigorously to form callus cells.

An elaborate seminal root culture system allows us to study epithelial cell activity that is quite different from that which takes place during germination. This system maintains the metabolic activity of scutellar cells for a prolonged period. After excision of the plumule and endosperm from a germinating seed, an isolated seminal root with the attached scutellum can be cultured under aseptic conditions utilizing two separate media: scutellum medium and root medium, as shown in **Fig. 1** (Radi and Maeda, 1986; Maeda and Radi, 1991).

The scutellum medium contains 12.5% sucrose, and is supplied to the seminal root via the scutellum, while the root tip is immersed in medium containing Murashige and Skoog's (MS) inorganic salts. During incubation, the volume of the scutellum medium gradually increases due to the excretion of metabolites from the scutellum, while the root medium decreases through absorption by the growing seminal root. Interestingly, ninhydrin-positive substances (amino acids and peptides) are found in the scutellum medium after growth of the seminal root.

The ultrastructure of the scutellar cells in this system was examined during incubation and compared to that in germinated seeds. The ultrastructural features were remarkably different in these two conditions. In the germinated seeds, almost all the constituents of epithelial cells were completely consumed within two weeks of germination. In the root culture system, on the other hand, the scutellar cells remained healthy for more than three months. Mitochondria and starch grains remained abundant, vacuoles were small, and sometimes there was a highly developed endoplasmic reticulum, although little cell-division activity was seen (Radi and Maeda, 1987).

Next, we developed a method to make a callus from the scutellar epithelial cells. A sterile culture of mature seeds on solid-agar medium containing a high concentration of 2,4-dichlorophenoxyacetic acid (2,4-D 10^{-5} M) produced large callus masses, revealing that scutellar epithelial cells retain dividing activity. There was no cuticular layer on the callus surface, since the callus originated from embryo cells lacking a cuticle.

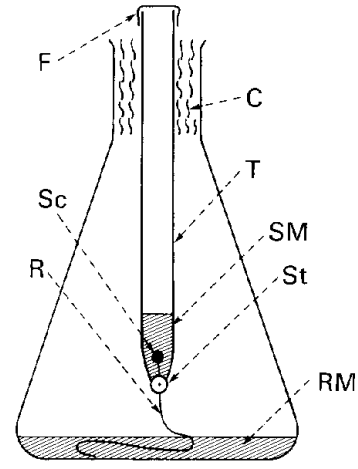

Fig. 1 Diagram illustrating the method used to culture a rice seminal root using two separate media simultaneously. C, cotton; F, aluminum foil; R, seminal root; RM, root medium; Sc, scutellum; SM, scutellum medium; St, stopper; T, glass tube.

Gross morphology

Laetsch (1971) reported that the tissues of monocotyledons, especially grasses, are traditionally difficult to grow in culture. However, there were indications that many of these problems may be solved. In 1968, five institutes in Japan simultaneously published scientific papers on shoot-bud formation from rice callus, and since then hundreds of papers on rice callus culture have been published worldwide.

Rice callus can be produced from various seed parts on agar medium containing modified MS basal medium, which is usually supplemented with 10^{-6} M 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, and 30 g l^{-1} sucrose.

Callus has been produced from the lateral root initials of seminal roots, the proximal ends of coleoptiles and young leaves, and vascular traces in the scutellum. However, most of the callus is formed from scutellar epithelium, and it can be grown into huge masses (Nishimura and Maeda, 1977). Shoot buds can be produced on the callus surface by transferring small pieces of callus into shoot-forming medium containing traces of 2,4-D (10^{-7} M) and high levels of kinetin ($5x10^{-5}$ M).

First, we offer here a brief overview of the gross morphology of the shoot-forming callus. During observations of the growth of callus on shoot-forming medium, four distinguishable morphological changes could be seen on the surface of the callus with the naked eye (Maeda *et al.*, 1986). They were the (1) S-surface (smooth surface), (2) N-surface (nodular surface), (3) H-surface (hairy surface), and (4) T-surface (protuberant surface), and they generally arose in that order. These morphological events could be distinguished by successive observations of the pattern on the callus surface at a given location.

When transferred to the shoot-forming medium, the callus surface appeared smooth or slightly undulating. By day 3 in culture, 87.5% of the callus still showed the S-surface, while the remaining cultures showed either the N- or H-surfaces. There was a gradual increase in the percentage of callus with the N-surface until 11 days after transfer, when a decrease occurred. Simultaneously, the percentage of callus possessing the H-surface increased until 24 days after transfer. During this period, the T-surface also appeared and increased from 9.0 to 43.1%. These morphological changes on the callus surface were sequential. All four surfaces were seldom seen simultaneously on the same callus. In some instances, the sequence was reversed.

Shoot buds always developed after the protuberant stage, nearly 2 weeks after the callus was cultured on shoot-forming medium. Thus, the proposed pathway that leads to shoot-bud formation is as follows: the S-surface changes to the N-, H-, and T-surfaces, in order, and finally, shoot buds develop. This indicates that a thorough understanding of the spatial and temporal patterns of the callus surface is essential to understanding shoot-bud formation.

Leafy appendixes are often seen on the protuberant surface. They develop directly from the callus surface or from the shoot apexes that are initiated on the protuberant surface. Scanning electron microscopy (SEM) reveals epidermal cells on the leafy appendixes, and a number of stomata and sickle-shaped trichomes that develop from the epidermis. However, the leaf shape generally differs from that of normal leaves on intact rice plants. Various abnormally shaped leaves are seen on plantlets that develop during callus culture. The morphological types include scale-like leaves, leaves with two or three tips at the terminal end, leaves with a shoulder at the right and left margins near the leaf tip, the absence of a ligule or auricles, and the appearance of a rudimentary ligule. Sometimes the ligule is divided into small pieces.

These abnormal leaves are usually seen in an assembly of leafy appendixes that develop directly from the callus surface. The abnormal leaves are always attached near the base of the shoot axis of regenerated plantlets, while normal-shaped leaves, arranged in normal order, are produced later at higher levels. This morphological change in successive leaves on the shoot axis or leafy appendix implies that there is a step during which an abnormal type normalizes to the normal type (Inoue and Maeda, 1981b). There are two routes to shoot-bud formation: embryogenesis and organogenesis. In embryogenesis, the first leafy appendix has a coleoptile-like structure, while in organogenesis a prophyll-like structure forms a tiller bud (Maeda, 2000).

Physiological gradient

Every cell in the entire callus is involved in a physiological gradient that depends on its spatial relationship to the solid medium. The cells in contact with the medium react to substances contained in the medium, while different responses probably occur in the cells located at a distance from the medium. The growth pattern of the entire

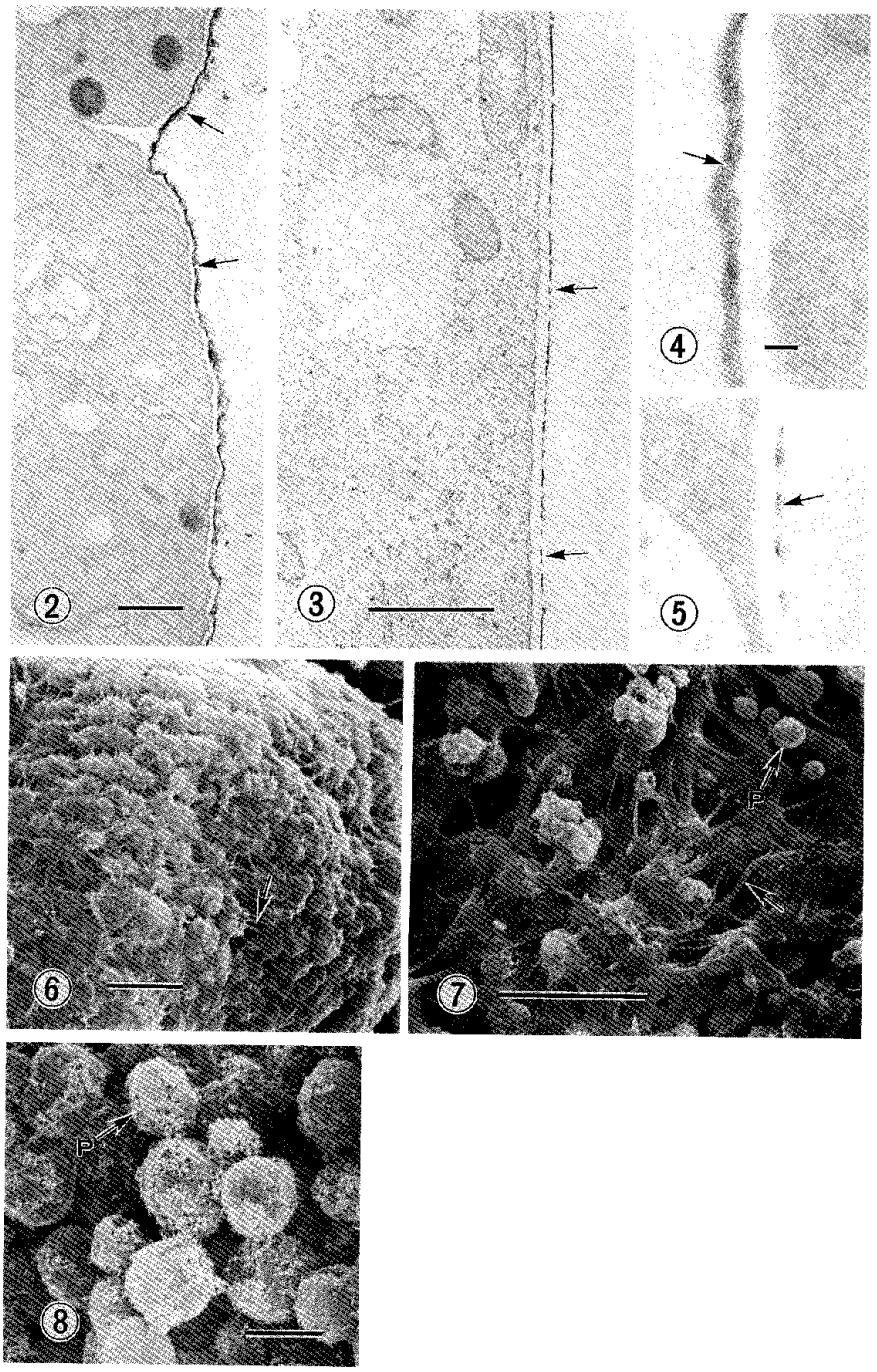

Fig. 2 Surface stratum of the dorsal scutellum in a rice embryo 4 days after anthesis examined by TEM (bar: 1 μm). Arrows show a continuous electron-dense layer of cuticle.

Fig. 3 Surface stratum of the dorsal scutellum in a rice embryo 5 days after anthesis examined by TEM (bar: 1 μm). Arrows show a discontinuous electron-dense layer of cuticle.

Fig. 4 Continuous layer of cuticle on surface stratum of the ventral side of a rice embryo 5 days after anthesis revealed by silver proteinate method (bar: 0.1 μm). An arrow shows a continuous layer.

Fig. 5 A discontinuous layer of cuticle on surface stratum of the dorsal side of a rice embryo 5 days after anthesis revealed by silver proteinate method. An arrow shows a discontinuous layer. The magnification is the same as in Fig. 4.

Fig. 6 SEM image of rice callus surface showing fibrils (arrow) interconnecting the ruptured membranous layer (bar: 60 μm).

Fig. 7 SEM image of the callus treated with cell wall digesting enzymes for 1 hr. Note the presence of undigested fibrils (arrow) and the release of protoplasts (P) (bar: 60 μm).

Fig. 8 High magnification of protoplasts (P) released from the callus surface (bar: 10 μm).

mass is determined by the combination of these responses.

In spite of the predicted relationship between callus growth and shoot-bud formation, to date there has been limited success in describing the detailed growth pattern of calluses. This is partially because there is no suitable method of following callus growth and recording the growth pattern. Furthermore, the growth pattern differs with the plant species studied.

Van Lith-Vroom *et al.* (1960) tried to trace callus growth in *Rubus fruticosus*, *Vissus* sp., *Helianthus tuberosus*, and *Daucus carota*. First, they covered the entire surface of the initial explants with charcoal powder and cultured the explants on solid medium. After callus growth, the distribution of the powder on the surface and within the callus mass was recorded and growth patterns were estimated based on the localization of the powder. As a result, species differences in the growth patterns were recognized.

A paraffin sectioning method was used to elucidate the anatomical details of tobacco callus. As positional markers, a few resin granules were placed on the surface of the shoot-forming medium on which the callus was cultured. Blocks containing the resin granules and an entire callus were embedded in melted paraffin wax in order to preserve the orientation of the callus tissue. Observation of microtome sections clarified that the anatomical features of each part of the callus differed, depending on the distance from the medium (Maeda and Thorpe, 1979); the lower part of the tobacco callus accumulated starch grains more rapidly than the upper part. Pronounced starch accumulation occurred in the parts protruding into the medium, and shoot buds were formed on the surface of these protrusions. Consequently, a physiological gradient concept was used to determine the loci where shoot primordia would be organized.

The physiological gradient in a tobacco callus is disrupted by inversion treatment, which involves inverting the callus on the agar medium. When the callus is inverted after 3, 6, or 9 days in culture on shoot-forming medium, the number of shoot buds is drastically reduced and shoot buds emerge from both the new and old basal parts of the callus (Ross and Thorpe, 1973). In rice callus, repeating the inversion treatments at short interval delays shoot formation and there is a marked decrease in the number of buds (a personal communication from Dr. M. Inoue). The physiological nature of each cell in a callus mass is likely determined by the distance from the point of contact with the medium and by the length of time in a particular orientation.

These results further support the diffusion gradient concept of organized development. The mode of absorption of nutritive substances from the medium differs between callus cells in contact with the medium and cells at a distance from the medium.

A growing rice callus is pale yellow and the callus surface is covered with an amorphous matrix. The matrix is wet, as a result of the movement of nutrient solution from the solid medium along the callus surface. Nutrients are supplied to the upper part of the callus from the medium by the effect of surface tension. Since the position of each cell in the callus mass differs, cell behavior consequently varies. For example, cells become dividing cells, parenchymatous cells, or cells that absorb nutrients directly from the medium, depending on their position.

As a callus grows, new growth patterns gradually appear as a consequence of the mode of nutrient absorption via the newly formed callus. Consequently, white patches appear in the surface stratum of the callus. Such a callus becomes more compact and appears drier in response to the new physiological gradient. When the auxin concentration in the medium is increased, these white patches tend to occur on the upper part of the callus. If the compact area including a white patch is transferred to shoot-forming medium, shoot buds are formed at fairly high frequency.

Two-step culture system

Sufficient control of shoot-bud formation in rice callus cultures can be achieved by using a two-step culture method. This method is also an excellent system for exploring the roles of phytohormones added to the medium, and for analyzing the mechanism underlying shoot formation. The two-step system consists of a preculture and a final culture.

In the first step, rice callus is induced from mature seeds on medium containing high levels of 2,4-D, and is then subcultured on the same medium to allow multiplication of the callus masses. These are further subcultured for about 30 days on medium containing high levels of 2,4-D and various phytohormones. This subculture step is called the preculture step for shoot-bud initiation.

Next, the callus is cultured on different media to promote organ development. This step is referred to as the final culture. The medium for the final culture contains little (10^{-7} M) or no 2,4-D. Cytokinins are added to the final culture medium to promote shoot formation, although when added to the preculture they are either ineffective or repressive in shoot

formation.

The mode of morphogenesis is clearly determined by the kind and concentration of phytohormones added to the preculture and final culture medium (Inoue and Maeda, 1981a; Kobayashi *et al.*, 1992).

When callus grown on preculture medium containing 10^{-4} M abscisic acid (ABA) is transferred to final culture medium containing kinetin, shoot formation is promoted and plantlet regeneration occurs. It has been suggested that the first step in shoot genesis starts during the preculture, and not in the final culture, where shoot buds appear. Therefore, this culture system separates shoot genesis into two steps: a step responsive to ABA, and one responsive to cytokinins.

When ABA is added to the preculture medium, the callus weight and water content are lower than in non-ABA medium. The greater potential for shoot-bud formation with ABA is correlated with the dry appearance and compact texture of the callus. White and green patches are often seen on the surface stratum of high-potential callus. The potential for shoot formation is realized during the final culture, where the number of white and green patches increases considerably. Shoot formation occurs much more readily when a small piece of callus containing white patches is removed from the preculture callus mass and transferred to final culture medium containing cytokinins (Higuchi and Maeda, 1990).

The early stage of shoot formation with ABA might imitate the process of rice caryopsis ripening, during which the level of endogenous ABA initially increases and then gradually decreases. Starch grains are frequently stored in the cells near the white patches. In this context, it is interesting that the level of endogenous ABA falls as starch accumulates in rice caryopsis.

A preculture medium containing mannitol or higher concentrations of sucrose (6-9%) is also effective in promoting shoot formation. When the sucrose concentration is 6%, instead of the usual 3%, callus with a dry appearance and with white patches is produced, as in ABA application. Similar results are also obtained with medium containing both 1.63% mannitol and 3% sucrose. The osmolarity of this medium is 282 mOsm, which is nearly equivalent to that of 6% sucrose medium. By day 7 in subculture on such media, a reduction in callus growth occurs. Dry, compact white patches appear on the callus (Higuchi and Maeda, 1991a). Presumably, the water stress imposed by the osmotic conditions plays a role in the formation of the white patches on the callus surface.

The effect of sucrose has been demonstrated in other japonica (Tsukahara *et al.*, 1996) and indica (Brisibe *et al.*, 1990) rice cultivars. With some indica-type rice, a high frequency of shoot formation is obtained from the callus, which has, inherently, a low water content. When the water content of the callus is decreased in medium containing high concentrations of sucrose or mannitol, the rate of shoot formation increases (Lai and Liu, 1988). Moreover, dehydrating callus by placing it on sterile dry filter paper for 24 h before transferring it to shoot-forming medium also increases the frequency of shoot formation (Tsukahara and Hirosawa, 1992) and the germination rate of somatic embryos (Mariani *et al.*, 2000). From this, it is concluded that the water stress induced by ABA, mannitol, excess sucrose, or dehydration increases the shoot-forming competence of rice callus remarkably.

Regeneration syndromes

The external morphology of rice callus can be friable or compact. Vigorously growing callus derived from japonica-type rice cultivars is characterized by a friable texture with a damp appearance and a pale yellow color. Shoot buds are produced at a rather low frequency on this type of callus. However, when transferred to shoot-forming medium, areas with peculiar morphology often arise on the callus surface. The first peculiar feature was small green patches on the callus, the surface of which generally appeared dry (Nakano and Maeda, 1979). In rice (Inoue and Maeda, 1980) and *Avena* (Heyser and Nabors, 1982; Nabors *et al.*, 1982) calluses, it was revealed that the number of green patches has a positive and quantitative relationship to the number of induced shoot buds.

If a well-growing callus is subcultured on medium with added ABA, the growth rate of the callus decreases slightly, producing a compact callus with white patches. When small pieces containing the white patches are transferred to shoot-forming medium, assemblages of green spots are frequently induced and leaf primordia develop near these areas (Inoue and Maeda, 1980; Higuchi and Maeda, 1990, 1991a). ABA also induces shoot buds even when the callus is cultured in liquid medium (Kobayashi *et al.*, 1992).

A decrease in the rate of callus growth is occasionally related to the appearance of a brown colored area. When excess cytokinin is added to the medium, these brown areas become larger. An increase in the number of brown areas and the development of an intense brown color results in a decrease in the number of shoot buds that form.

When a well-growing callus is repeatedly subcultured on medium containing a high level of 2,4-D, the callus becomes more friable and damp in texture, resulting in a low frequency of shoot formation. After 7 or 8 successive subcultures, the callus is extremely friable and shoot buds rarely form, even when the callus is transferred to shoot-forming medium.

To clarify the underlying mechanism of shoot formation, it is necessary to examine the variation in the surface stratum and texture. The culture conditions that promote shoot formation should be able to transform a wet, friable callus into a dry, compact one. A compact callus generally possesses white or green patches and, occasionally, slightly necrotic areas.

In addition, the occurrence of a cuticle layer over a limited area of the surface stratum and the differentiation of a vascular bundle system inside the callus are correlated with the formation of leafy appendixes and shoot buds.

Since there is an intimate relationship between the water stress and shoot bud formation, it is reasonable to compare *in vitro* shoot formation with the ripening of rice caryopses. Therefore, an optical microscope study (OM) and TEM of zygotic embryogenesis in rice were conducted (Suzuki *et al.*, 1996), and moreover, the location of phytin particles on rice caryopses has been studied.

In the shoot apical meristem region of rice embryos, transcripts of myo-inositol hexa-phosphate synthase were detected by *in situ* hybridization (Yoshida *et al.*, 1999). This enzyme is involved in the biosynthesis of phytic acid. Interestingly, this synthase was isolated from embryogenic rice callus. Phytic acid is made up of salt deposits containing cations (Mg^{2+}, K^{+}, etc.). These deposits are stored inside spherical phytin particles located in protein bodies or small vacuoles. These particles stain red to purple-red with toluidine blue O, and are called globoids histologically. Small globoids have been detected in several cell regions near the shoot apical meristem. Thus, the localization of globoids might match the activation pattern of the enzyme gene in rice embryos.

The size and frequency of globoids are positively related to phosphorus content in various crop seeds (Wada and Maeda, 1980). Wada and Lott (1997) examined the composition of globoids in protein bodies located in various tissues in rice embryos in detail using energy dispersive X-ray microanalysis. Furthermore, phytin particles were temporarily present in small vacuoles in the cells near the meristematic center in shoot-forming rice callus, and in the distal region of roots, pollen grains, flower buds of rice and vegetables (**Table 1**), and winter buds of cherry trees (a personal communication from Dr. T. Wada). Consequently, phosphorous and other metal ions, and carbohydrates temporarily accumulate near the predetermined shoot meristem in rice callus and are subsequently utilized for shoot development. Once again, these results show that the mode of shoot formation in rice callus is similar to the zygotic embryogenesis of rice from the viewpoint of phytin accumulation.

Various morphological changes can be perceived by the naked eye on callus that gives rise to shoot-buds. The callus is dry and compact in appearance, with white and green patches, and brown necrotic areas. The positions of these patches are relative to the location of the solid medium. We propose that these signs are called regeneration syndromes. The cuticular layers on the callus surface, the phytin particles near the meristem, and the starch grains that accumulate at particular loci should be included in this classification after a thorough cytohistologic and electron microscopic study.

In the calluses of indica-type rice, the pattern of patches differs with the cultivar used, because there is a large amount of variation in the gross morphology of these calluses (Maeda, 1967a, b). A rice callus is not merely an aggregate of homogeneous cells, but consists of cells with different properties. Consequently, biochemical analysis of shoot formation is often affected by the dilution effect of mixing unessential parts with the samples examined. Fur-

Table 1 Relative peak of each metal relative to phosphorous in phytin particles in the inflorescences and root cells of different plant species (data from T. Wada).

Materials	P	Mg	K	Ca	Mn	Fe	Zn
Rice pollen	10	(1)	(1-3)	4-6	(1-2)	-	(1-3)
Rice flower bud	10	1-3	(2-4)	1-10	(1-2)	(1)	(1-3)
Broccoli flower	10	1-3	3-5	1-8	-	-	(1)
Maize root	10	2-3	3	2-3	-	-	1-3
Pea root	10	2-3	4-5	-	-	-	1

( ): Sometimes detected.

thermore, at present it is difficult to specify a target cell or cell groups within the huge mass of a callus.

The success of *in vitro* shoot formation depends on the phytohormones added to the media, the concentrations used, and the timing of hormone addition. Despite the fact that we paid attention to these factors, our experiments did not always succeed. This was largely due to a lack of precise knowledge of the regeneration syndromes. It is necessary that the culture condition is correctly determined on the basis of the temporal and spatial changes in the callus surface. This is especially important in recalcitrant plants. Therefore, to obtain callus competent for shoot formation it is necessary to obtain a topographical and temporal description of the true picture or virtual form of the growing callus. New topographical images of the callus surface need to be made using optical and electron microscopy, and computer simulation models.

Microtopography

Land plants need to conserve water in their tissues under a dry air environment. In principle, the inner tissues are surrounded by epidermal cells, which are coated with water-impermeable fatty cuticles. However, some plant parts, such as the root caps, have a mucilaginous coating consisting of glycoproteins and polysaccharides. On the other hand, seaweed has surface cells possessing a mucilage-secreting ability (Juniper and Jeffree, 1983).

The epicuticular surface of plants is formed only at the early stage of zygotic embryogenesis; if this surface is accidentally removed, it will not be regenerated, except in aseptic callus cultures (Bruck and Walker, 1985a, b; Walker and Bruck, 1985).

The outer surface of a growing rice callus is not covered with a compressed rigid wall, but is mostly surrounded by a mucilaginous matrix moistened by the culture medium (Maeda, 2000). The surface architecture of the globular, compact callus is modified by small white-colored patches, which are dry in appearance and are partially covered with cuticular layers.

Although knowledge of regeneration syndromes allows us to notice the significance of topographical variation in the efficient regeneration of shoot buds, not enough studies of the ultrastructural changes during *in vitro* shoot formation have been undertaken. The undoubted importance of such studies is further supported by SEM studies. SEM images of rice callus are useful for evaluating the various morphological events that are a prerequisite for shoot-bud formation.

When seen with the naked eye, the surface of the pale yellow callus appears smooth, but SEM examination shows that it is rough. By contrast, the surface of callus with white patches appears rough to the naked eye, while it is smooth by SEM. Thus, SEM images, which focus on a limited area, produce a very different view from the wide image obtained by the naked eye.

The mucilaginous layer covering the pale yellow surface is fragile and easily torn. The superficial cells can be seen often underneath a ruptured membrane. This rupture probably results from the fast growth and rapid division of the subsurface cells. In addition, fibrils are seen near these ruptures and interconnect the ruptured membranes (**Fig. 6**). Cellulose microfibrils are also observed in the transition period from pro-embryos to globular embryos during somatic embryogenesis starting from immature zygotic rice embryos (Mariani *et al.*, 1998). It has been suggested that this fibrillar material is transformed into the cuticular layer (Mariani *et al.*, 1999).

Mucilaginous substances excreted from the superficial cells are used to repair the broken layer. The hydrophilic mucopolysaccharides in this fragile layer actually give the callus surface its wet appearance. **Fig. 9** shows the PAS-positive matrix of rice callus. The wet matrix layer of a growing callus is unstable.

It has been reported that *Viola patrinii* plantlets are often regenerated from green compact callus, but not from light-brown friable callus (Sato *et al.*, 1995b). Wheat and sweet potato calluses are surrounded by a thick layer of moist matrix, and results in a lower ratio of shoot formation. On the other hand, an epicuticular layer develops over a limited area of the outer surface of compact rice callus. With prolonged culture, the superficial cells covered by this layer elongate slightly, take on the same orientation, and are transformed into protodermal cells. Finally, the surface stratum of these cells is stabilized by maturation of the epicuticular layer.

When the cuticular layer is formed in the mucilage matrix of rice callus surface, the movement of nutrient solution through the mucilage matrix is probably disrupted at the cuticle-deposit area. With the difference of light reflection, as a result, the outward appearance of the area will be perceived like dry. The leafy appendixes, shoot apexes, and shoot buds arise from these areas.

Fig. 10 is a putative diagram of shoot-bud formation. It illustrates the sequence of events preceding formation of a shoot apical meristem in rice callus. The first step is the growth of a friable callus, which is completely enveloped by a mucilaginous matrix that is moistened by the culture solution (**A**). Next,

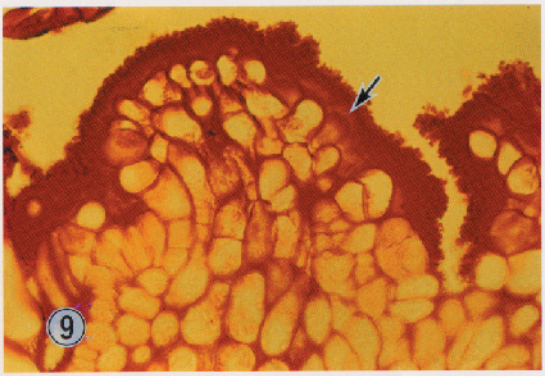

Fig. 9 A longitudinal section of callus stained by the PAS method showing the presence of mucilaginous matrix (arrow). (This micrograph was taken by M-H. Chen).

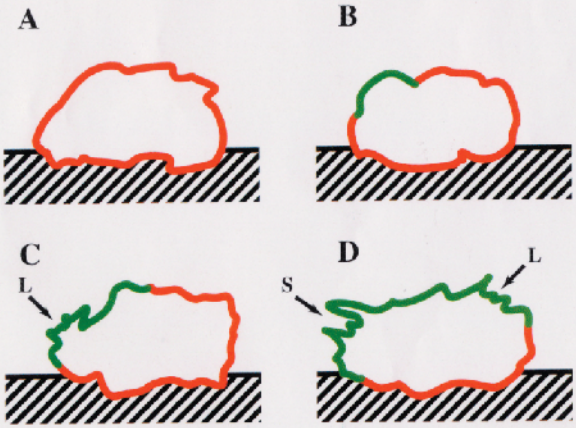

Fig. 10 Diagram showing the hypothetical sequence of events leading to shoot-bud formation in *O. sativa* subsp. japonica. In the longitudinal figures of the callus, the red regions contain mucilaginous matrix and the green color shows the presence of a cuticular layer. Leafy appendixes (L) and a shoot bud (S) develop in the green region.

dry, white areas appear on part of the callus surface, together with an epicuticular layer coated with water-impermeable cutin materials, and some superficial cells are transformed into the protoderm (**B**). Subsequently, outgrowths that look like leafy appendixes occur in these areas. They often have anomalous leaves, but lack shoot apical meristems (**C**). Finally, a shoot apical meristem forms on the epicuticular layer, and a shoot develops with an orderly arrangement of successive leaf (**D**). These developmental processes are supported by the fact that the shoot apical meristem of the rice embryo is formed on the ventral side, where the cuticular layer is initially formed.

An experiment was performed to clarify the surface architecture and inner structure using identical callus samples. Pieces containing white patches were first examined by SEM and then by OM. Using this method, tracheary elements were detected by OM in the leafy structure verified by SEM examination, while no shoot apical meristem was seen near this area (Higuchi and Maeda, 1991b).

Vascular elements are scattered throughout the interior of a growing callus, while a callus mass that gives rise to shoot buds contains regularly arranged vascular bundles running in one direction that include xylem and phloem elements.

Studies of coffee callus have helped us to understand the extracellular matrix over rice callus. Coffee callus can be produced from explants excised from leaf blade. A whitish-cream colored callus proliferates from the cut edges of the leaf blade. Initially, a fast-growing, soft callus forms, which is soon followed by the formation of a dark black callus. After prolonged culture, a small, pale-yellow embryogenic callus appears on the dark callus, and sequential events leading to coffee embryo formation are shown (Nakamura *et al.*, 1992).

SEM examination reveals a globular somatic embryo with a well-defined suspensor, a friable yellowish callus, and a brownish callus. The brownish callus consists of elongated cells covered by a membranous layer. A glycocalyx layer of PAS-positive mucilage is evident on both coffee callus and coffee somatic embryo. A membranous layer generally covers the callus surface. Where this membrane is ruptured, a rough callus surface is exposed. The presence of a thin membranous layer covering the superficial cells of coffee callus, and their rupture with the growth of a bulky callus, are events similar to those seen in rice callus (Nakamura and Maeda, 1990).

Mucilaginous materials cover both the suspensor of somatic embryos and the embryonic callus. However, the presence of a cuticular layer can be clearly detected by the silver proteinate method on the surface of globular embryo. A high magnification shows the initial cell of secondary embryo in the suspensor region of the primary embryo. Mucilaginous material is seen on the entire surface of the early secondary embryos. The basal cells of primary embryo are covered with an external coat of mucilaginous material, but the coat gradually thins towards the top, where a cuticular layer is formed.

Consequently the development of somatic embryo from coffee callus is associated with the transformation of the mucilaginous matrix to cuticular membrane lined with a compressed wall (Nakamura *et al.*, 1994). In the early development of carrot embryos, Lackie and Yeung (1996) described how cuticular materials are deposited on the outer tangential wall of protoderm cells, but not on the cell wall of suspensor.

Extracellular matrix on the callus surface

The polysaccharide-coated surface of plant cells is called the glycocalyx. The nature of the glycocalyx of higher plant cells changes as the cells age, and differs from cell to cell within the plant body. The cell itself synthesizes the glycocalyx as a functional surface in bacterial, animal, and higher plant cells (Costerton *et al.*, 1978). Carpita *et al.* (1996) described the relationship between the extracellular matrix and wall formation in plant cells.

The existence of proteins in the mucopolysaccharide was proven by the partial dissolution of the extracellular matrix of *Cichorium* callus by proteinase (Dubois *et al.*, 1992). A fibrillar network linking the superficial cells in this callus was also detected.

When plant tissues enveloped in epidermis are incubated with wall-degrading enzymes (pectinase and cellulase), the epidermal cells are hardly digested because they are covered with chemically stable epicuticular materials. Rice protoplasts have been successfully isolated from chopped sections of roots and leaves, but not from the epicuticular surface (Maeda, 1971; Maeda and Hagiwara, 1974). Sato *et al.* (1995a) isolated numerous protoplasts from chopped petunia leaves and rice callus, and reported a practical method of fixing isolated protoplasts for SEM examination.

When the surface of rice callus is treated with wall-degrading enzymes, two distinctive features are revealed on the callus surface: a membranous layer and fibrillar structures. The enzymes digest the membranous layer, while the fibrillar structures

are not affected. The membranous matrix is PAS-positive and mucilaginous. Protoplasts are released from the areas where the mucilaginous layer was enzymatically digested.

Sato *et al.* (2001) carefully removed the entire callus mass from culture flask, while retaining its shape, and incubated it without shaking in an enzyme solution containing 1% macerozyme R-10, 4% cellulase Onozuka RS, 10 mM $CaCl_2 \cdot 2H_2O$, and 0.4 M mannitol (pH 5.8). Before and after the enzyme treatment, the coat matrix of the callus surface was examined with SEM and OM. After a 1-h incubation, the extracellular matrix of the callus surface was digested and the PAS-reactive polysaccharides were selectively removed. As a result, the superficial cells were found by removing the outer matrix, and many protoplasts were released from the digested areas (**Fig. 8**). This proved that the extracellular matrix consists mainly of enzyme-digestible mucous materials.

The surface morphology differed in large, old callus. Many fibrils connected the edges of the ruptured matrix layer (**Fig. 6**). The fibrils remained after removal of the mucous matrix by enzyme treatment (**Fig. 7**). Two hours after starting the enzyme treatment, many more polysaccharides were removed, and the matrix was also severely digested. Consequently, many gaps appeared among the callus cells, and additional protoplasts were released. Even after a prolonged incubation in the enzymes, the fibrils persisted on the callus surface. Many protoplasts were released from the sites where there were no fibrils.

Gas chromatographic analysis of the mucilage extracted from soybean callus indicated the presence of glucose and galactose (Keese *et al.*, 1991). Honda *et al.* (1997) showed that glucuronomannan polysaccharides, major components of the extracellular material, were deposited in the intercellular spaces of the surface cells in a tuberose callus mass. Šamaj *et al.* (1995) reported that a highly developed extracellular matrix was located on the surfaces of sundew and maize calluses. The proteinaceous substances were involved in the matrix, and bridges of net-like materials were inserted between the cells. Furthermore, arabinogalactan proteins with a high water-retention capacity were detected on the outermost walls of embryogenic cells in maize callus (Šamaj *et al.*, 1999a) and their localization was demonstrated by an immunogold SEM technique (Šamaj *et al.*, 1999b).

As the rice callus grows, fibrils appear in the mucilaginous matrix. The time of fibril appearance probably corresponds to the appearance of the white patches on the compact callus. The fibrils are made of a substance that is resistant to the action of pectinase and cellulase. The coating matrixes covering the callus cells differ with growth stage and location. They include mucilaginous material, fibrils, and an epicuticular layer. These structures can be observed separately using SEM. The spatial and temporal changes in the surface of rice callus are of interest in determining the mechanism of shoot-bud formation.

Conclusions

This review examined the morphological events that occur before establishment of the shoot apical meristem on rice callus cultures. Callus initiation and development of the shoot apical meristem have a spatial interrelationship deduced from the dorsiventrality of young rice embryos. Callus tissue derived from the dorsal surface lacks an epicuticular layer. The external appearance of callus depends largely on the extracellular matrix. Growing callus is covered with a mucilaginous matrix that is susceptible to wall-degrading enzymes. The physiological gradient and organizing polarity of the callus mass are determined by both the length of culture and the spatial position relative to the solid medium. Conditions that impose water stress on the callus mass increase the potential for plantlet regeneration. The shoot apical meristem forms on the epicuticular layers that arise at particular loci on the callus, which appear in a manner that resembles zygotic embryogenesis. These details suggest that the topographical changes in the callus surface and the biochemical changes in the coat matrix need to be clarified in order to maximize plantlet production. *In vitro* plantlet production is of practical importance in economically important plants. However, plant callus culture is not merely a technique for plantlet production, as it also involves basic problems in developmental biology. Morphological studies of callus cultures at macroscopic and microscopic levels should make a great contribution to advances in this field. Furthermore, knowledge of plantlet production is useful for dissecting the mechanisms of developmental processes such as zygotic embryogenesis and the orderly organization of tiller initials.

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