Ultrastructural Changes Leading to Plantlet Differentiation in Callus Cultures of African Rice

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Abstract : Semithin and ultrathin sections of callus tissues of African rice (*Oryza glaberrima* Steud) after 7 and 14 days of initiation were compared with similar cultures after 3, 5, 7 and 10 days of incubation on a differentiation medium. Histological examinations indicated that at the early stages of development, both types of callus tissue appear structurally similar with small, almost isodiametric and meristematic cells located toward the callus periphery. In addition to this cell type, cultures on a differentiation medium also contained large parenchymatous and highly vacuolate non-differentiating cells. Ultrastructurally, while the former were characterized by a relatively large number of organelles, the non-differentiating cells contained only a thin peripheral layer of cytoplasm. Later in culture, the differentiating cells undergo periclinal divisions resulting in the formation of radial files of tissue which correspond to the meristematic regions where shoot primordia differentiation is generally initiated. There is also evidence to show that the differentiation of shoot and/or root primordia in callus tissues is a high energy demanding process. This energy requirement is fulfilled by the accumulation, mobilization and utilization of starch and other reserve substances prior to the onset of plantlet regeneration.

Key words : Amyloplast, Callus, Differentiation, *Oryza glaberrima*, Plant regeneration, Ultrastructure.

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要 旨 : 誘導後 7 日目および 14 日目のカルスと、再分化培地に移植してから 3, 5, 7 および 10 日目のカルスの組織を光学顕微鏡および電子顕微鏡で比較した。培養初期にはいずれのカルスも、周辺部にほぼ等直径の分裂組織細胞が観察されたが、再分化培地のカルスでは内部に液胞化した大型の柔細胞が存在した。分裂組織細胞には多数のオルガネラが観察されたが、内部の柔細胞には周辺部にのみ細胞質が存在した。培養日数が経過すると再分化過程の細胞は並層分裂を繰り返し、放射状の細胞列からなる分裂組織を形成し、この中より茎葉が発生した。再分化過程の分裂組織のプラスチドは一時的に多量のデンプンを蓄積した。またクリステの発達したミトコンドリアや、液胞中での物質分解が観察された。従って茎葉分化過程は高エネルギー要求過程と考えられ、デンプンその他の貯蔵物質の蓄積、移動、分解によってまかなわれると考えられた。
キーワード : アフリカネ、アミロプラスト、カルス、再分化、植物体再生、微細構造変化。

In vitro plantlet regeneration is of special significance not only in cereal crop breeding, where culture manipulation and genetic transformations could be effected, but also in developmental biology where it serves as an ideal experiential system to investigate morphogenetic and ultrastructural changes underlying the dynamics of cell and plant differentiation. On the basis of these advantages, the *in vitro* culture response of virtually all the major graminaceous species has been evaluated with

some degree of success²⁰⁾. In most of these species, including rice, an improvement in culture conditions and medium manipulations has led to the differentiation of plantlets, either through somatic embryogenesis^{5,20)} or organogenesis^{2,7,8,10)}. However, in spite of these investigations, knowledge of the specific mechanisms underlying the differentiation of plantlets through either of these developmental processes remains abysmally inadequate.

Lack of detailed structural information describing the initial processes leading to the differentiation of *in vitro* plantlets in rice (and other major cereal crops) is surprising, considering that these are the mainstay of the world's food economy. Thus far, at least to our knowledge, only the studies of Nakamura and Maeda¹⁰⁾, Maeda et al.^{7,8)} and Brisibe et al.²⁾

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have focused on the sequence of morphogenetic events, which result in the onset and initial stage of shoot and root primordia development in callus cultures of *Oryza sativa* and *Oryza glaberrima*, respectively. Of particular interest in these studies is the scanning electron microscope indication that plantlets were differentiated via non-synchronized, independent development of shoot primordia. These reports also demonstrated that the presence of a stabilized callus surface layer actually triggered the initial processes of plantlet regeneration^{2,8)}.

Collectively, although these studies have increased our general understanding of the developmental events during *in vitro* morphogenesis in rice callus cultures, they did not precisely characterize the cytological events associated with the developmental switch from the non-differentiated state to that of plantlet differentiation at the cellular and subcellular levels. It is the purpose of the present study to establish this developmental correlation between structural alterations observed earlier on callus surfaces (see Brisibe et al.²⁾) with functional aspects of cellular and subcellular changes leading to the differentiation of shoot primordia in African rice.

Materials and Methods

1. Callus induction and culture conditions

In the current study only one variety of African rice-IRRI accession number 101853 was used as plant material, because it was earlier observed to produce both the highest callusing ability and plantlet regeneration frequency²⁾.

Callus was induced from mature caryopses on modified MS medium containing dicamba ($30\text{ }\mu\text{M}$), L-proline (12.5 mM), casein hydrolysate (3 g/l) and solidified with 0.2% Gelrite. Cultures were initiated on similar

conditions as those described previously²⁾.

Plantlet differentiation studies were evaluated on a shoot forming medium that was supplemented with $0.5\text{ }\mu\text{M}$ dicamba or $0.01\text{ }\mu\text{M}$ 2, 4-D and $5\text{ }\mu\text{M}$ kinetin, on ceramic wool liquid culture as already described²⁾.

2. Light and transmission electron microscopy

For light microscopy, callus tissue on an induction medium after 7 and 14 days of explant incubation and callus fragments of differentiating cultures at 3, 5, 7 and 10 days after transfer to the plantlet regeneration medium, were fixed at 4°C for 4 to 6 hours in glutaraldehyde buffered at pH 7.0 with 0.1 M sodium cacodylate buffer. The samples were thoroughly washed and rinsed with several changes of the buffer and post-fixed for 2 hours in 1% aqueous osmium tetroxide. They were dehydrated in an ascending ethanol series and propylene oxide, embedded and polymerized in an epoxy resin. To make semithin sections for light microscopy, they were cut on a Reichert-Nissei Ultracut N ultramicrotome at a thickness of $0.5\text{--}1.5\text{ }\mu\text{m}$, dried onto glass slides and stained with 0.5% (w/v) aqueous toluidine blue for routine general observations.

For developmental studies at the ultrastructural levels, ultrathin sections were obtained for transmission electron microscopy using diamond knives at a thickness of 80–90 nm from the materials embedded for light microscopy. They were collected on copper grids, stained with 5% uranyl acetate in 1% acetic acid and post-stained with lead citrate. Sections were examined and photographed with a Hitachi H-600 transmission electron microscope at 100 kV.

Results

Like those of *Oryza sativa*, light microscope observations of explants at inoculation in-

Explanation of Figures

- Fig. 1. Light micrograph of embryonal scutellar epithelium showing elongated and dividing cells (Sc) after 3 days of culture. Note the meristematic nature of scutellar cells ($\times 60$).
- Fig. 2. Differentiation of superficial callus layer (Ca) ($\times 80$).
- Fig. 3. Transmission electron micrograph of callus cells showing the presence of large, spherical and centrally located nuclei in the callus cells. Cells have thin walls with plasmodesmata (Pd) and vacuoles (V) ($\times 2800$).
- Fig. 4. Transmission electron micrograph showing the presence of many conspicuous amyloplasts containing starch grains (S) ($\times 6400$).

Fig. 5. High magnification of a typical amyloplast containing starch (S). Some microtubules (Mts) can also be observed ($\times 20000$).

Fig. 6. Transmission electron micrograph of callus cells after 14 days of culture on induction medium. A prominent and large nucleus (N) containing nucleolus (Nu) can be observed. Cells also have many plasmodesmata (Pd) ($\times 6400$).

Fig. 7. Light micrograph of epoxy resin-embedded semithin section of African rice callus on differentiation-promoting medium. Division of callus tissue into two prominent histological regions : a peripheral or outer region (PR) and an inner region (IR) are clearly observed. Note the differentiation of meristematic cells, which are small and electron dense, in the peripheral region. Also note that cells of the inner region are big, parenchymatous and non-dividing. Small radial files of tissue indicated with small arrows can be observed on the lower left hand corner ($\times 100$).

Fig. 8. Magnification of an amyloplast observed in differentiating cells at 3 days after transfer. The characteristic pattern of arrangement and localization of starch grains (S) appear to indicate that they are undergoing some hydrolysis (indicated with arrows) ($\times 9600$).

Fig. 9. Some notable ultrastructural features of the differentiating cells at 3 days after transfer. The characteristic presence of plastids undergoing deformation can be observed. Also note the presence of some cytoplasmic components in the vacuole (*) ($\times 16000$).

Fig. 10. Parenchymatous cells from the non-dividing region of callus tissue. Most of the cells have large vacuoles appressing the entire cytoplasmic matrix of each cell into a thin peripheral layer (*) next to the cell wall ($\times 4800$).

Fig. 11. Differentiating cells showing characteristic presence of endoplasmic reticulum with some ribosomes ($\times 4800$).

Fig. 12. A portion of a differentiating callus cell showing the conspicuous presence of a chloroplast (C) containing some starch grains (S). Chloroplast matrix cannot be seen clearly right now ($\times 16000$).

Fig. 13. Differentiating cells after 7 to 10 days of transfer to the regeneration medium showing large numbers of rough endoplasmic reticulum. Note the presence of ribosomes ($\times 36000$).

dicated that the scutellar epithelium of the mature embryo of African rice appears as a modified layer of columnar cells at the interface of the embryo with the endosperm. These cells have a prominent central nucleus, numerous peripherally localized lipid bodies, and small mitochondria with ill-defined cristae¹³. Within 2 to 4 days of inoculation on the callus induction medium the epithelial cells have elongated (Fig. 1). By 5 to 7 days, a superficial callus layer had developed (Fig. 2). To the inner side of the callus layer, meristematic activity and callus development accelerated, resulting in massive growth of callus tissue after 14 to 21 days of culture.

1. Ultrastructure of callus cells on induction medium

The ultrastructural cytology of cultures at 7 days after incubation of explants on the induction medium indicated that callus cells exhibited distinctly meristematic characteristics: dense and granular cytoplasm with large centrally located spherical nucleus containing large nucleoli and only little heterochromatin (Fig. 3), several small vacuoles and mitochondria. In these cells endoplasmic reticulum profiles were rather variable, but plasmodesmata were frequent in walls between adjacent cells.

A prominent ultrastructural feature of these cells was the characteristic presence of numerous amyloplasts (Fig. 4), which contained few or several starch grains that occupied the entire organelle (Fig. 5). Microtubules and a sparse number of microbodies and lipid bodies were also isolatedly observed. By 14 days of culture, callus cells have become more electron dense and prominent. Golgi bodies and numerous mitochondria could also be observed (Fig. 6).

2. Ultrastructural changes leading to plantlet regeneration

Light microscopy of the differentiating cultures on a plantlet regeneration medium showed that callus was composed of two histological zones: (i) a central or inner region; and (ii) a peripheral or outer region, which were greatly diverse in cellular size, degree of vacuolation and differentiation. On account of this division callus cells at the central or inner region were observed to be highly vacuolated and non-dividing while those of the peripheral region were small, almost isodiametric and meristematic (Fig. 7). These eventually developed to produce radial files of tissue at the callus periphery.

Transmission electron microscopy amplified the separation between meristematic and non-meristematic regions of the callus tissues earlier identified by light microscopy. Ultrastructurally, as the transfer of callus pieces from the induction (after 30 days of culture) to the regeneration medium resulted in a series of complex and interrelated cellular changes prior to and during the initial stages of shoot primordia differentiation, description of the entire process is provided after the following durations:

(1) 3 days after transfer

At this stage of development meristematic regions of differentiating callus tissue consisted of relatively small vacuoles and thin walls. These cells also had many mitochondria, Golgi bodies and short profiles of endoplasmic reticulum. Figure 8, which is a higher magnification illustration of a typical amyloplast shows the arrangement and localization of starch grains in the cells. Other notable ultrastructural features of these cells include plastids undergoing deformation (Fig. 9), and vacuoles containing some cytoplasmic compo-

Fig. 14. Ultrastructural illustration of differentiating cells after 7 to 10 days of transfer to the regeneration medium showing conspicuous mitochondria with well developed cristae and Golgi bodies ($\times 16000$).

Fig. 15. Ultrastructural depiction of a portion of non-differentiating cell with characteristic localization of multimembranous structures (indicated with arrows). The functions of these features are not properly understood ($\times 9600$).

Abbreviations used in Figures

A : amyloplast, CW : cell wall, ER : endoplasmic reticulum, G : Golgi body, M : mitochondrion, N : nucleus, P : plastid, S : starch/starch grain, V : vacuole.

nents (indicated with asterisk in Fig. 9). In contrast, the non-meristematic regions had large and thick walled parenchymatous cells (Fig. 10). Most of these cells were generally highly vacuolated, often with only a narrow peripheral region of cytoplasm, typically containing a large number of mitochondria. In general, these cells appeared variable in size and shape, did not exhibit any features that could be associated with active proliferation and were therefore possibly non-totipotent.

(2) 5 days after transfer

By day 5 the early developmental phases leading to plantlet differentiation had commenced, with the formation of radial files of meristematic tissue as depicted by the light microscopy shown in Fig. 7. It is apparent from this micrograph that continued division of the superficial meristems may have led to the nodular appearance observed on callus surfaces (as earlier described in Brisibe et al.²⁾). Such rapid and continued divisions may have also resulted in the formation of the epicuticular layer on callus surfaces²⁾.

Ultrastructurally, the differentiating cells had changed dramatically. At this stage the cell cytoplasm appeared more electron-dense, containing small lipid bodies but starch deposits had progressively decreased. Other conspicuous differences noted between the meristematic cells at 5 days compared to those at 3 days involved the prominence of large amounts of endoplasmic reticulum (Fig. 11) and large numbers of mitochondrial profiles with dilated cristae. The vacuoles also contained some membrane fragments.

Over and above these general observations the cells at 5 days also appeared especially active, containing prominently large nuclei. Often the differentiation of chloroplasts from plastids could also be observed (Fig. 12). Chloroplast matrix was not quite visible. Chloroplasts also appeared flattened and contained only few starch granules.

It is significant to note that macroscopically at this stage most of the callus pieces had become chlorophyllous, leading to the formation of green and pigmented spots on the lower portions in contact with the culture medium. This observation is intriguing since it would be recalled that the chlorophyllous development of callus (that is, green spot formation) at this stage had been reported

earlier²⁾.

Some amyloplasts containing starch could be isolatedly observed in some of the non-meristematic parenchymatous cells.

(3) 7 to 10 days after transfer

After 7 to 10 days in culture, when the developmental process had actually proceeded either to the primordial stage, or was actually characterized by the formation of many leafy vegetative shoots on the callus surface, differentiating cells presented a structural profile similar to those leading to shoot production from a normal meristem. Most of the meristematic cells were rich in their internal constituents; having large amounts of rough endoplasmic reticulum with ribosomes (Fig. 13), conspicuous mitochondria with internal cristae (Fig. 14), large population of Golgi bodies and centrally located nuclei with one or two nucleoli. Interestingly, by 7 to 10 days after inoculation on the differentiation medium lipid bodies and starch deposits had completely disappeared from the cells.

Another difference of still greater significance at this stage was the striking structural diversity between superficially exposed cells and those of the underlying layer. Whereas up till 5 days, the degree of vacuolation in superficial cells had only little differences from those of the substratum, there was a marked variation by 7 to 10 days. At this stage vacuoles of the superficial cells enlarged, often through the coalescence of two or more smaller ones. Nucleus and cytoplasm remained conspicuous and some multivesicular membranes (Fig. 15) were also observed in some of the cells. In more differentiated portions of the cultures adjacent cells exhibited common characteristics typical of companion cells such as high numbers of mitochondria and many plasmodesmata.

For comparison, the ultrastructure of non-differentiating cells homologous in orientation and location to those of the earlier developmental stages was also examined. Quite often the tonoplast of parenchymatous cells had deteriorated. As with earlier stages, they had big vacuoles that occupied the entire central portion of the cell. The cytoplasm and nucleus were peripherally located; appressed to the cell wall as a result of the occupation of the greatest part of the cell volume by the vacuole. Although a large number of mitochondria in

the cytoplasm and multimembranous structures in vacuoles were observed, no starch deposits or other storage reserve materials could be identified.

Discussion

Light microscope observations of differentiating calli at early developmental stages clearly demonstrate that the periphery of the meristem is composed of highly ordered radial files of tissue with tangentially oriented cell divisions. The appearance of these files of tissues surely is in good agreement with corresponding structural data achieved by earlier studies using scanning electron microscopy²⁾ and, hence can be correlated to the compact and nodular nature of callus at early stages of differentiation. Furthermore, since the changes in cell expansion towards the callus periphery are mediated by rapid and continued division of these files of tissue, it is conceivable that this can also be taken as evidence of their role in the formation of the epicuticular layer and trichomes, as earlier observed on callus surfaces²⁾.

Transmission electron microscopic examinations of meristematic cells have also disclosed new details in the pattern of cytological events leading up to the differentiation of shoot and/or root primordia involving cell size, population of organelles, cytoplasm and vacuolation. Evidently, the current results permit an unambiguous interpretation of cellular differentiation and microtopography of the superficial meristem, which correlates with and extends our earlier findings in callus cultures^{2,10)} using scanning electron microscopy. Particularly interesting in the present study is the comparison of the cellular states of differentiating cells at early stages with those immediately prior to or during the initial stages of plantlet differentiation, which showed that major differences were observed in the organization of the cell types.

At later stages of differentiation some of the most obvious features of these developmental changes involve the prominence of centrally located nuclei and mitochondrial profiles with conspicuous cristae, the expansion of vacuoles and the dramatic disappearance of starch and other reserve storage materials after 7 to 10 days of culture. Also of great significance at this developmental stage is the observation of

large numbers of Golgi bodies, free ribosomes and endoplasmic reticulum which appeared typically dilated. It is conceivable that these ultrastructural features are consistent with changes common to plant cells undergoing higher metabolic activities; including the synthesis of proteins and enzymes such as found, for example, during high growth rate and differentiation³⁾. This corroborates earlier biochemical findings in *Oryza sativa* showing significantly increased RNase and α -amylase activities at more advanced stages of plantlet regeneration in callus cultures¹⁶⁾. It is likely that some of these enzymes, especially α -amylase triggers the hydrolysis of starch and other reserve storage materials in the cells, presumably in keeping with the role of those metabolites during the early stages of plantlet differentiation.

Another significant developmental correlation noted in this study between changes on callus surface and cellular activities involve the dramatic disappearance of starch from the differentiating cells at 7 days and beyond. This coincides with the period during which leafy vegetative shoot formation occurs on callus surface as earlier evidenced by scanning electron microscopy²⁾. This is also the time when maximum rates of increase in callus fresh weight occurs¹⁸⁾. The loss of starch from differentiating cells in callus tissues has been noted in other investigations^{6,17)}. Carbohydrate residues released by the hydrolysis of starch grains could provide the initial energy required for the differentiation of shoot primordia, as also noted in differentiating cells of tobacco^{14,19)}, *Solanum aviculare*¹⁾, carrot⁴⁾ and *Begonia rex*⁹⁾.

Finally, we should like to comment on the suggestion that starch present during the growth and development of differentiating cells may be indicative of some embryogenic developmental capacity^{12,15)}. This might be a simplistic, though fairly well understood view since the presence of starch in embryogenic cells is interpreted as morphological evidence of their high nutritional requirements necessary for somatic embryo development¹¹⁾. However, it is difficult to reconcile this view with the presence of starch in organogenic callus^{6,14,18)} as well as the high density of starch-laden amyloplasts noted in the present study. Perhaps, the implication of these observations

is that though the morphological development of starch may differ in the two developmental processes (that is, somatic embryogenesis and organogenesis), its role seems the same in principle. This view agrees with previous studies which have demonstrated that starch metabolism plays a significant role in cellular events leading to plantlet differentiation through organogenesis^{14,18}). It is likely that this also seems to be the case with the African rice cultures in the current study, where the general decline in starch following periclinal divisions (see Results) fits with the conclusion that the accumulation, mobilization and utilization of starch in differentiating cells are necessary prerequisites for the initiation and development of shoot and root primordia.

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