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Enhanced Plant Regeneration in Rice Callus Cultures Following Abscisic Acid Treatment

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Abstract : Several changes were shown in rice callus cultures with abscisic acid (ABA) treatment. First, callus growth was inhibited, especially at a high concentration (2×10^{-4}M) of ABA. Secondly, white, dry and compact regions were formed in the callus at higher frequency. Thirdly, two kinds of callus structures were observed at ABA-treated culture stage with a scanning electron microscope (SEM). White regions and yellow regions at macroscopical level corresponded to relatively smooth, undulated structures and rough-surfaced structures, respectively at SEM level. Moreover, the dark condition in which ABA was treated enhanced the frequency of appearance of white regions in the callus and advanced the developmental stage leading to plant regeneration.

When the calli having white regions were predominantly transferred on the regeneration medium, they showed the highest potential to regenerate plantlets. Nevertheless, the ability of such regions to regenerate plantlets was not affected by illumination in the ABA-treated culture at all. Several morphological types in plant regeneration processes could be observed after 7 days of the regeneration culture. Some of them showed ordinary organogenesis and others malformed processes.
Key Words : Abscisic acid, *Oryza sativa*, Plant regeneration, Scanning electron microscopy, Tissue culture.

アブシジン酸前処理によるイネカルス再分化の促進：樋口暢宏・前田英三（名古屋大学農学部）

要　旨：アブシジン酸（ABA）を前培養の段階で与えると，イネカルスに次に挙げるような変化が起こることがわかった．まず第一にカルス生長に対して阻害作用が認められ，それは特に高濃度(2×10^{-4}M)のABAの場合に，顕著であった．第二に，処理したカルスが白色及びコンパクトな乾燥状態になった．第三に，処理カルスを走査電子顕微鏡（SEM）レベルで観察したところ2種類の構造，つまり比較的滑面のこぶ状の構造と，カルス表面上にあった膜構造が破壊されつつあると思われる構造が認められた．さらに，ABA処理を暗条件で与えると，第二に示した白色構造の出現頻度が上昇した．またABA処理したカルス塊をその形態的特徴から選別して，再分化培地に移植すると，ABAを前培養時に処理した際に生じた白色部位が非常に高い再分化能力を有していることも明らかになった．しかしABA処理時の光条件は，白色部位の形成頻度には影響を及ぼすが，植物体再分化能力の点からみた白色部位自体の性質には変化を及ぼさなかった．実験で得られたカルス塊の中で最も高い再分化率を示したABAを処理した際に生じた白色部位カルスを，再分化培地に移植してから7日目の状態をSEM観察したところ，通常の器官形成パターンによる再分化が多く確認されたが，それとは別の分化パターンも観察された．しかしこれが体細胞胚発生によるものであるかどうかは不明である．
キーワード：アブシジン酸，イネ，植物再生，走査電子顕微鏡観察，組織培養．

Application of *in vitro* techniques to plant improvement will depend on the regenerative capacity of certain economical important species. Although cereals have not been suitable species for plant regeneration, great progress has been made in recent years.

In rice as well as in other plants, genotypes and explant sources were often considered to be important factors in determinating plant regeneration. For example, a relationship between plant genotype and *in vitro* response, particularly regeneration, has been reported in several cereal species such as rice[1,11,29,40], oat[9] and wheat[13]. Contrary to these results, we have found many reports where plant regeneration were obtained in most of the genotypes tested. Moreover, they also showed the significance of physiological and developmental state of the explants. For instance, somatic embryogenesis of cereal plants have been obtained from the specific developmental stages of embryos [13,18,20,26,36], inflorescences [3,6,7,28,36] and leaves[12,19,39]. Vasil[35] discussed that genotypic effect is strongly influenced by physiological and environmental factors, which in turn influence the synthesis, transport and the availability of phytohormones. This argument is strengthened by the fact that highly embryogenic explants and calli showed higher levels of both indole-3-acetic acid (IAA) and ABA[27].

Inoue and Maeda[14] presented the two-step

culture method by ABA to enhance the regenerative capacity of rice callus cultures. Abscisic acid is generally known to be inhibitory to plant growth and antagonize the stimulatory effect of other hormones, auxins and cytokinins. However, less information is available on the stimulatory effects of ABA on plant regeneration. Kochba et al.[16] and Vasil and Vasil[37] demonstrated that embryogenesis is stimulated by ABA. Shepard[31] and Torrizo and Zapata[34] observed ABA-promoted shoot regeneration in potato and rice callus cultures, respectively. In addition, Abou-Mandour and Hartung[2] showed the stimulation of adventitious root formation in *Zea mays* callus cultures.

In the present report, we study the effect of ABA on the growth and differentiation of rice callus cultured *in vitro*, especially the morphological effects leading to plant regeneration by SEM observation.

Materials and Methods

Husked mature seeds of *Oryza sativa*. L. (var. Nipponbare) were surface-sterilized with 70% ethanol for 3 min, followed by 5% sodium hypochlorite solution for 25 min and then rinsed with sterile distilled water several times. The culture medium designated here as a basal medium was consisted of Murashige and Skoog's major and minor salts[23], 78.4 mg/*l* FeEDTA, 200 mg/*l* myoinositol, 1 mg/*l* thiamine-HCl, 3 g/*l* casein hydrolysate, 3% sucrose and 0.65% INA-agar (BA-10). Sterilized seeds were placed on the callus induction medium (basal medium plus 10^{-5}M 2,4-D) and inoculated for about 30 days. Calli induced from scutellum were subcultured on the same medium for 25 days. The purpose of this subculture is to minimize heterogeneity of the callus. Subcultured calli were inoculated on the preculture medium (basal medium plus 10^{-5}M 2,4-D and various concentrations, 2×10^{-4}, 2×10^{-5} or 2×10^{-6}M of ABA) for 25 days. After this preculture, the calli were transferred on the regeneration medium (basal medium plus 10^{-7}M 2,4-D and 5×10^{-5}M kinetin).

All cultures were kept in a culture room at 25～27°C under continuous light of about 3,000 lux except for the dark condition of the examination on illumination.

For scanning electron microscopy, the materials were fixed with modified Karnovsky's fixative[15] consisted with 3% glutaraldehyde, 1.5% paraformaldehyde and 0.1M Sörensen's phosphate buffer, by gently shaking at a room temperature for 5 h. The materials were rinsed repeatedly with the above buffer and dehydrated with graded ethanol series. The dehydrated materials were treated with isoamyl acetate and critical point dried with HITACHI HCP-1. They were coated with gold by an ion sputtering equipment, EIKO IB-3 and examined with a scanning electron microscope, HITACHI S-415.

Results

We have examined the effect of ABA in the preculture medium on rice callus growth, the morphology and the ability to regenerate plantlets. A preliminary experiment has indicated that the callus growth as measured by fresh and dry weights, decreased with increase in ABA concentration from 0 to 2×10^{-4}M (data not shown). Furthermore, this inhibition of callus growth was always accompanied by the appearance of particular regions with both white and dry characters in the callus about 15 days after transfer on the medium with higher concentrations of ABA (Figs. 2 and 4). The white and dry regions were frequently observed near brown necrotic regions and had many starch grains through histochemical investigations (data not shown). On the other hand, when cultured on the medium either without ABA (Figs. 1 and 3) or with low concentration of ABA, i.e. 2×10^{-6}M, the calli were usually yellow and wet in appearance, and the formation of white and dry regions was rarely observed.

Table 1 shows that the highest potential for plant regeneration was obtained from the preculture medium supplemented with 2×10^{-5}M ABA, at which concentration green spots and adventitious buds were observed earlier than those at other ABA concentrations tested. At 2×10^{-4}M ABA, necrosis pronouncedly appeared on the callus. Consequently, this high level of ABA resulted in low frequency of plant regeneration.

As mentioned above, the optimal concentration of ABA in the preculture medium is 2×10^{-5}M. Moreover, as ABA-treated calli were characterized by the intensive formation of white and dry regions, we have investigated

Table 1. Effect of abscisic acid concentration in the preculture medium on regeneration from rice callus.

ABA conc. in preculture (M)	% of calli forming adventitious buds	% of greening calli
0	14.3	31.4
2×10^{-6}	14.0	26.0
2×10^{-5}	20.0	30.0
2×10^{-4}	12.0	22.0

Callus was cultured on the preculture medium supplemented with 10^{-5}M 2,4-D and various concentrations of ABA, and transferred to the regeneration medium with 10^{-7}M 2,4-D plus 5×10^{-5}M kinetin. Data were collected after 25 days culture on the regeneration medium.

Table 2. Effect of selective transfer according to color on regeneration from rice callus.

Preculture ABA (M)	color	% of calli forming adventitious buds	% of greening calli
0	yellow	20.3±4.3	39.1±2.6
2×10^{-5}	white	51.6±2.6	60.4±5.5
2×10^{-5}	yellow	9.4±0.6	22.6±2.5

Callus was cultured on the preculture medium supplemented with 10^{-5}M 2,4-D and various concentrations of ABA, and transferred to the regeneration medium with 10^{-7}M 2,4-D plus 5×10^{-5}M kinetin. Data were collected after 25 days culture on the regeneration medium. Selection with color was made macroscopically at the end of preculture. On control, the calli cultured on a medium without ABA, were not selected at all. ABA concentration in the preculture medium was 2×10^{-5}M. S.E. is indicated.

the ability of the calli having such regions to regenerate. After 25 days of inoculation on the preculture medium with or without 2×10^{-5}M ABA, the calli were selected according to the difference in color and transferred on the regeneration medium. The calli treated with ABA could fall into the following two categories; (A) "ABA white callus" characterized to be white, opaque and dry in nature, (B) "ABA yellow callus" of yellow, semitranslucent and wet in appearance. In contrast, since the calli grown on the preculture medium without ABA scarcely had the white and dry regions, only yellow regions of such calli were transferred in this case. In the present report, this type of calli is referred to as "control callus". Control callus is also yellow, translucent and wet, which nature is the same as ABA yellow callus macroscopically.

Table 2 demonstrates that ABA white callus had the highest percentage of both adventitious bud formation and greening. In addition, such percentages of ABA white callus were remarkably higher than those of ABA-treated callus without macroscopical selection (Table 1). However, no significant differences in regeneration activity were found between ABA yellow callus and control callus.

We have also studied the effect of illumination at ABA preculture stage on callus growth, its morphology and the regeneration activity. Table 3 shows that the callus growth was significantly inhibited with ABA treatment in the preculture medium, but not affected by illumination at all. However, more white and dry regions were formed on ABA-treated callus precultured in darkness as compared with those precultured in light. This superiority of darkness was remarkable even 10 days after inoculation on the preculture medium containing ABA when a few white and dry regions could be observed on ABA-treated callus precultured in light, whereas a considerable number of white and dry regions were already formed on ABA-treated callus precultured in darkness. As shown in Table 4, ABA white callus had a higher potential for regeneration, which precultured in light or in darkness, compared with control callus.

The calli on the preculture medium without ABA (control calli) were yellow and wet as indicated previously. When such calli were inoculated on the regeneration medium, the formation of white regions on the inoculated yellow calli frequently preceded plant regeneration. Then we have investigated the relationship between the formation of white regions on the yellow calli during the regeneration culture and their plant regeneration activity. Table 5 shows that the calli forming white regions during the regeneration culture were superior in plant regeneration to those remaining yel-

low.

As mentioned above, since ABA treatment induced changes in morphology and stimulated the formation of white and dry regions, we have examined what changes at the SEM level correspond to surface appearance at the macroscopical level. When cultured on the preculture medium without ABA for 25 days, a large proportion of the callus surface had a rough appearance (Fig. 5), although the calli having a smooth surface could be rarely observed (Fig. 6). In contrast, as shown in Figs. 7 and 8, we could observe the several types of structures on the ABA yellow calli at a high frequency, such as the thin membranous structures covered on the callus surface (Fig. 8), the radially directed fibrous structures (Figs. 7 and 8) and the exposed cells/cell aggregates (Fig. 8).

On the other hand, ABA white calli were characterized by smooth surface. Figs. 9—12 demonstrate several structures on ABA white calli. Some structures had a fold and undulated appearance, and others had specific characteristics such as tube-like (Fig. 11) or leaf-like (Fig. 12) structures. When calli were treated with ABA in darkness, we could observe the same structures, although the frequency of the formation of such structures in darkness was considerably higher as compared to that in light.

When the calli treated with ABA were transferred on the regeneration medium, several morphological types in plant regeneration processes could be observed after 7days of the regeneration culture. Fig. 13 shows the structure which was probably achieved by the enhancement of undulation, and its surface was similar to that of *in vivo* rice leaf blade. Furthermore, we could observe two leaf-like structures and three leaf primordium-like structures (Fig. 14) and some other malformed structures such as leafy structure with many trichome-like outgrowths and adjacent smooth nodular structure (Fig. 15) and symmetrical structure with hemispherical outgrowth (Fig. 16).

Discussion

In these present investigations, we have shown several changes in rice callus with ABA treatment. First, callus growth was inhibited, especially at a high concentration of ABA. Generally, the plant hormone ABA is known to be inhibitory to *in vivo* growth and its role in regulating growth has been reviewed[22]. Similar inhibitory effects of ABA has also been noted in several tissue cultures such as tobacco[17,41], *Lemna polyrhiza*[32] and *Ipomea*[30]. The present experimental result agrees to their reports. Contrary to these reports, we have found several reports where ABA treatment stimulates callus growth at appropriate concentrations[2,4,5,34].

Secondly, ABA treatment stimulated the formation of white, dry and compact regions in the callus. These characteristics are similar to those of so-called embryogenic callus[35]. In many species of the Gramineae, embryogenic calli are generally known to be hard, white, compact, dry and opaque. In addition, these calli consisted of small spherical and richly cytoplasmic cells. In the present experiment, light microscopic observation confirmed these histological characteristics in the ABA-treated calli. Moreover, when ABA white calli were predominantly transferred on the regeneration medium, they showed the highest potential to regenerate plantlets. Thus, these results indicate so-called embryogenic characteristics and potential of ABA white calli.

Thirdly, the dark condition in which ABA was treated enhanced the frequency of appearance of white regions on the callus and

Table 3. Effect of abscisic acid and illumination in preculture on rice callus growth.

Preculture		Fresh weight (mg)	Dry weight (mg)	Water content (%)
ABA (M)	Illumination			
0	light	334.9±13.7	29.6±1.3	91.2
2×10^{-5}	light	207.7±10.4	25.3±1.0	87.8
2×10^{-5}	dark	236.8±14.2	26.2±1.4	88.9

Data were collected after 25 days culture on the preculture medium. Fresh weight of initial inoculum was approximately 30 mg. S.E. is indicated.

Table 4. Effect of abscisic acid and illumination in preculture on regeneration from rice callus.

Preculture		% of calli forming adventitious buds	% of greening calli
ABA (M)	Illumination		
0	light	24.4	56.4
2×10^{-5}	light	57.7	87.2
2×10^{-5}	dark	61.5	80.8

Callus was cultured on the preculture medium supplemented with 10^{-5}M 2,4-D and various concentrations of ABA, and transferred on the regeneration medium with 10^{-7}M 2,4-D plus 5×10^{-5}M kinetin. Data were collected after 25 days culture on the regeneration medium.

Table 5. Relationship between the formation of white regions on the regeneration medium and plant regeneration activity.

Callus reaction on the regeneration medium	% of calli forming adventitious buds	% of greening calli
Remaining yellow	5.8±2.3	30.8±3.0
Turning white	52.6±7.5	73.2±14.0

Callus was cultured on the subculture medium containing 10^{-5}M 2,4-D for 25 days and transferred on the regeneration medium. When calli cultured on the regeneration medium, some of them formed white regions about 7 days after transfer (Turning white), others continued to be yellow during the last culture (Remaining yelllow). Data were collected at 25 days after culture on the regeneration medium. S.E. is indicated.

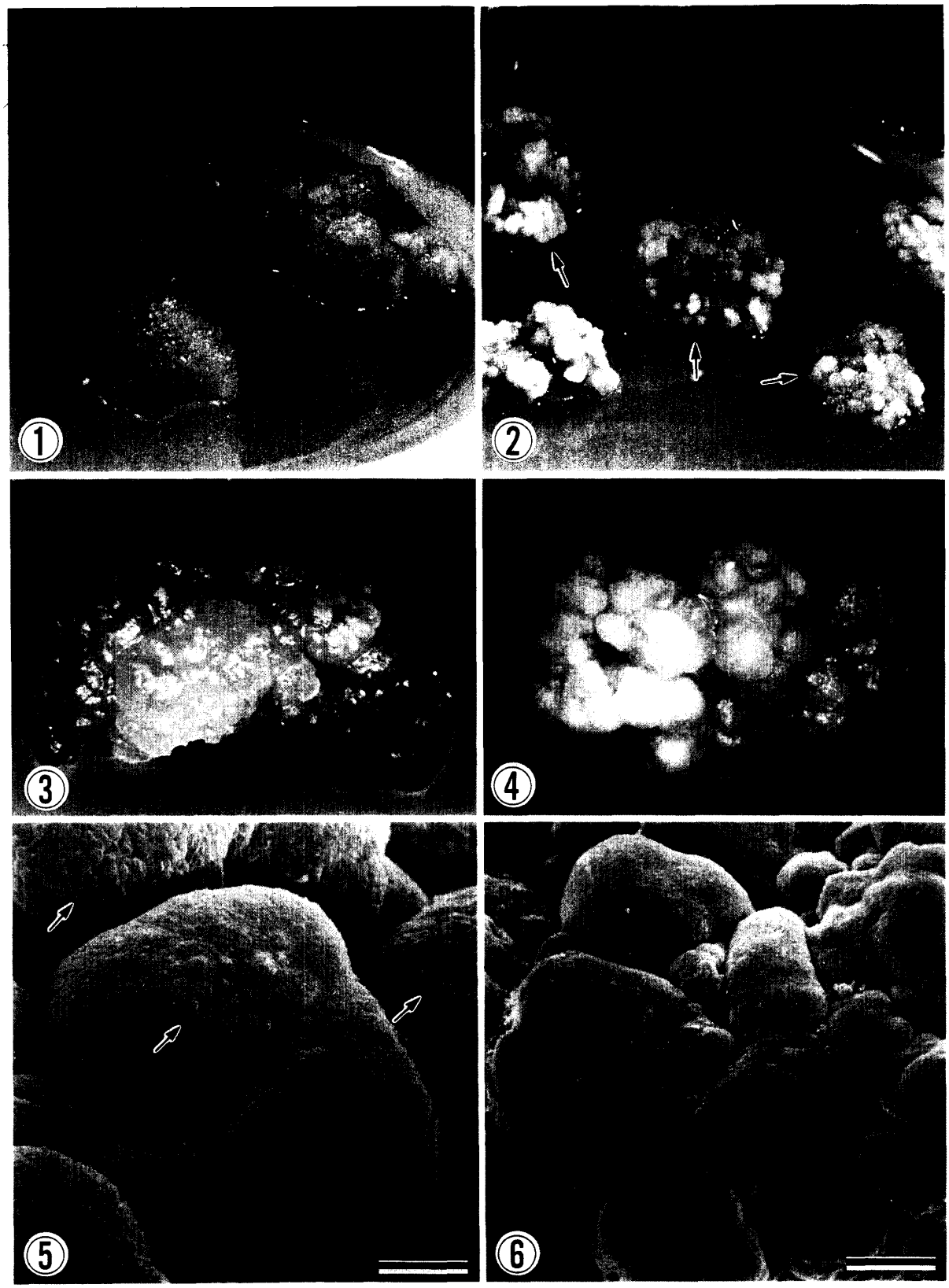
advanced the developmental stage leading to plant regeneration. The light condition, which is known to lower ABA level[10], may inhibit its stimulatory effect on the formation of white regions. However, we did not investigate endogenous ABA level at the preculture stage, it is not clear why the dark condition stimulated the formation of white regions. The dark condition enhanced the frequency of the formation of white regions; nevertheless the ability of such regions to regenerate was not affected by illumination in the preculture.

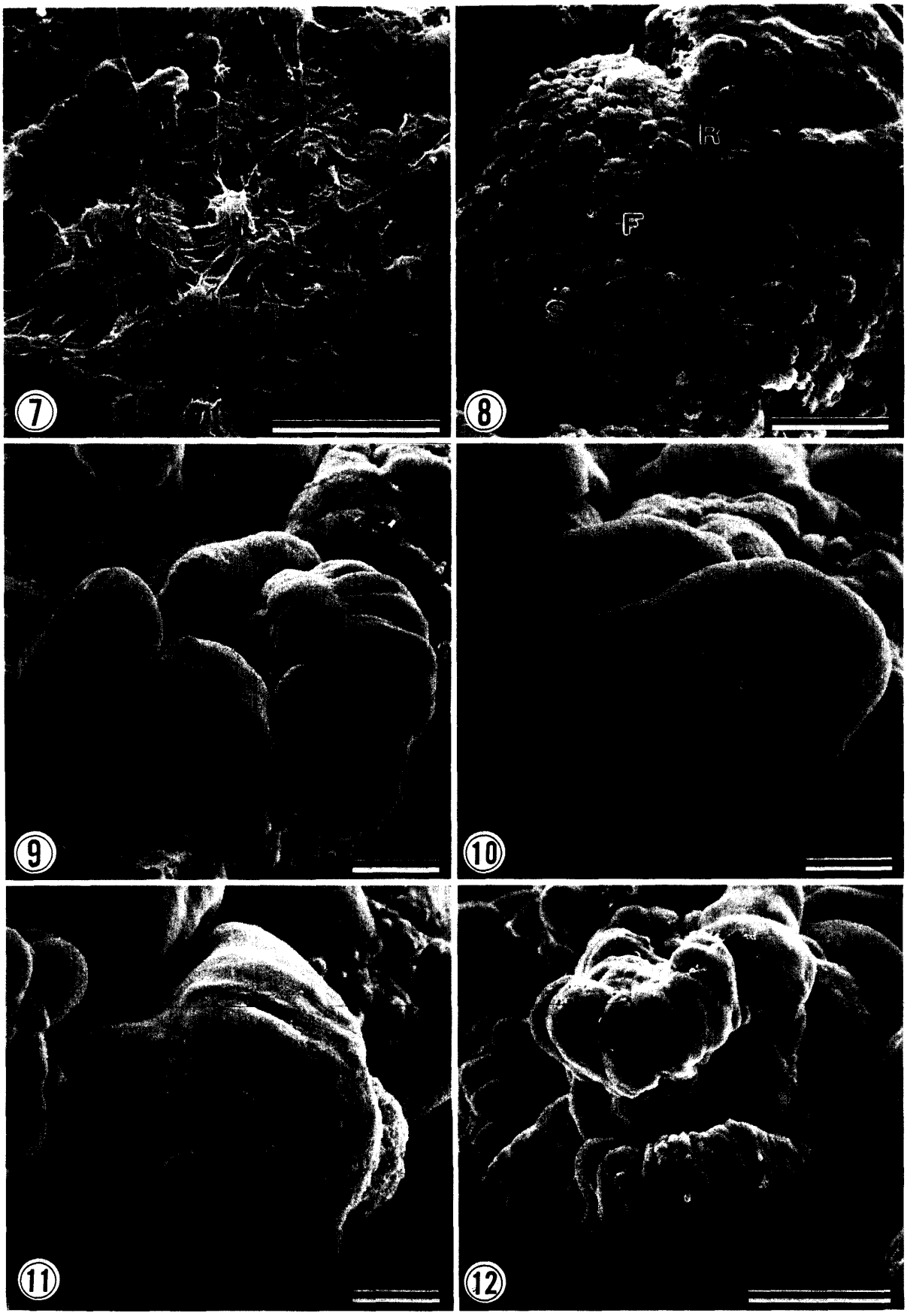
Furthermore, after the calli were inoculated on the preculture medium without ABA, some of them formed white regions during the regeneration culture, and they showed significant superiority in regeneration to those remaining yellow. Hence, the discoloration may be critical for plant regeneration. In rice tissue cultures[24], turning white of calli was found to precede adventitious bud formation. Lu and Vasil[19] and Botti and Vasil[6] reported that this white color is due to the accumulation of starch grains. We also confirmed the starch accumulation in the white regions of not only control calli but also ABA-treated calli. Thorpe and Meier[33] speculated such accumulated starch to be a readily available energy source during plant regeneration.

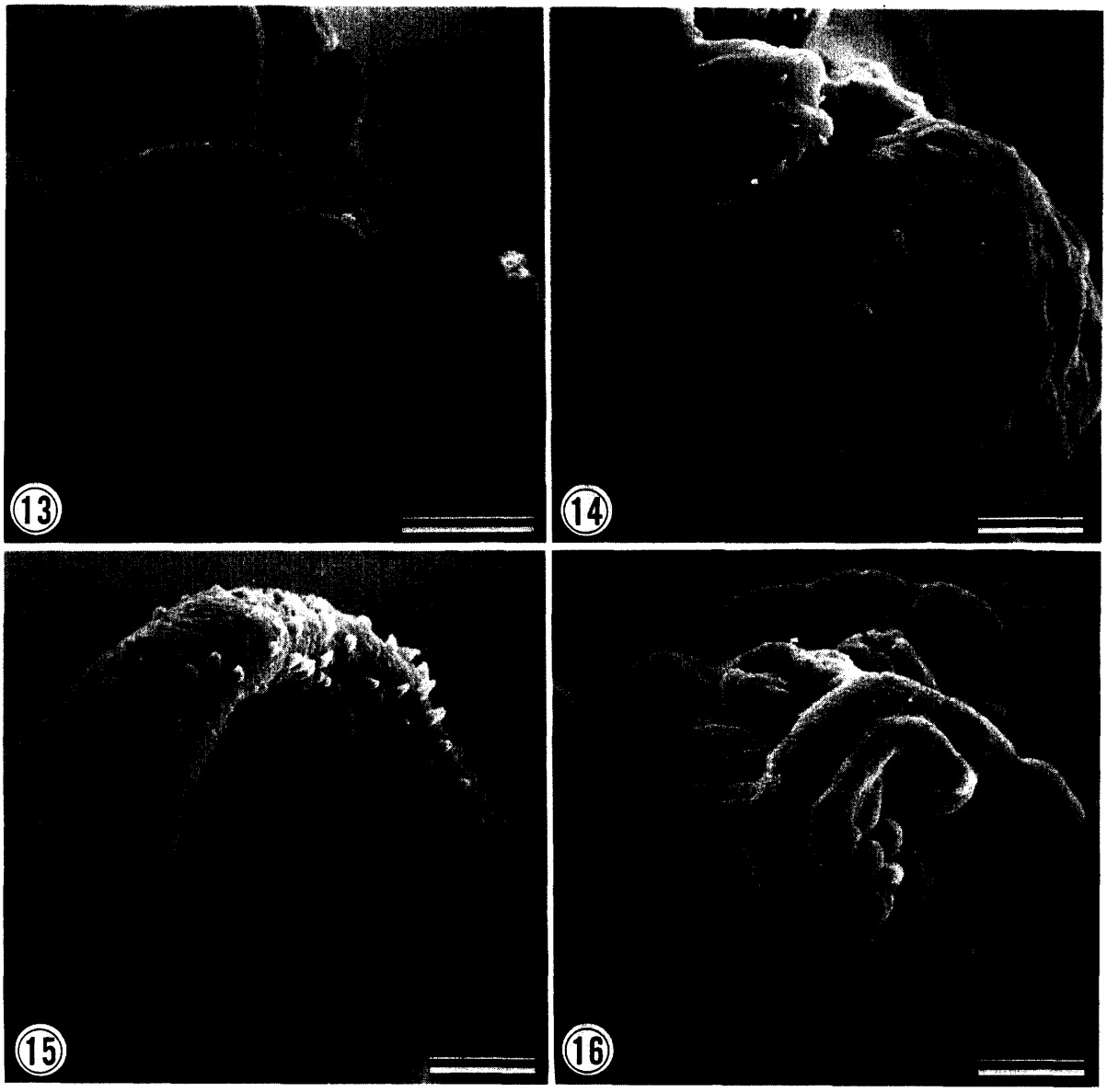
Th role of ABA in accumulating starch grains is not clear. Exogenous ABA was reported to induce the formation of white and opaque starch-containing callus in maize[8] and pearl millet[38]. In addition, it is well known that endogenous ABA level of seeds raises drastically at the early stage of embryogenesis and subsequently falls after starch accumulation in rice[25] and wheat[21]. Hence, ABA may play a role in accumulating starch by its inhibitory effect on biosynthesis of hydrolytic enzymes such as α-amylase. Apart from this speculation, Torrizo and Zapata[34], who reported the stimulatory effect of ABA on plant regeneration in *indica* rice callus, discussed the possibility that its influence in the accumulation of proline, which was assumed to stimulate plant regeneration in several species, however, the present experiment could not confirm this possibility.

We observed two kinds of callus structures at ABA preculture stage with SEM; White regions and yellow regions at macroscopical level corresponded to relatively smooth, undulated structures and rough-surfaceed structures, respectively at SEM level.

Several workers have shown the existence of superficial membranous structures, which are composed of polysaccharides. In our experiment, exogenous ABA promoted the disruption of such structures vigorously. In general, ABA is known to stimulate the secretion of several types of hydrolytic enzymes in *in vivo*







Explanation of Figures

Figs. 1 and 3. Calli grown on the preculture medium without ABA for 25 days.

Figs. 2 and 4. Calli grown on the preculture medium with ABA for 25 days. Many white and dry regions (arrows) are observed on the callus surface.

Figs. 5 and 6. Surface view of calli grown on the preculture medium without ABA. Rough-surfaced clumps (Fig. 5. bar 200 μm, arrows) and smooth-surfaced clumps (Fig. 6. bar 200 μm).

Figs. 7 to 12. Surface view of callus grown on the preculture with ABA. ABA yellow callus (Fig. 7. bar 50 μm and Fig. 8. bar 200 μm) showing rough-surfaced membranous structure (R), radial fibrous structure (F) and separated cells/cell aggregates structure (S). ABA white callus (Figs. 9 to 12) showing undulated structure (Figs. 9 and 10 bar 200 μm), tubular structure (Fig. 11. bar 200 μm) and wavy undulated structure and leaf-shape structure (Fig. 12. bar 300 μm).

Figs. 13 to 16. Surface view of differentiating callus structures on the regeneration medium for 7 days. Wavy undulated structures (Fig. 13. bar 200 μm), two leafy structures and three leaf primordium-like structures (Fig. 14. bar 200 μm), many trichome-like outgrowths and adjacent smooth nodular structure (Fig. 15. bar 100 μm) and symmetrical structure with hemispherical outgrowths (Fig. 16. bar 200 μm).

abscission. In the same manner, superficial membranous structures may break down with exogenous ABA treatment. However, appearance of smooth surface in white regions accompanied with rough surface in yellow regions suggests the participation of other factors such as differences of endogenous hormonal levels within one callus piece.

Smooth and undulated structures were observed on ABA white callus. They resembled embryogenic calli in their shape. In addition, when ABA-treated calli were transferred on the regeneration medium, several morphological types in plant regeneration processes could be observed after 7 days of the regeneration culture. Some of them showed ordinary organogenesis and others malformed processes (Figs. 15 and 16). However, we could not obtain any structures characteristic of embryogenesis. Thus, it is not appropriate to employ the terms "somatic embryogenesis" and "embryogenic callus" in this report.

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