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Plantlet Regeneration and Novel Protein Synthesis in a Long-term Cultured Callus of Rice in Response to Abscisic Acid

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Abstract : : Some physiological changes induced by abscisic acid (ABA) were investigated in a long-term cultured callus of rice, *Oryza sativa*, L. cultivar YUHKARA. The results were as follows : ① The retardation of callus growth was evident, while dry matter content was apparently increased with the addition of abscisic acid (ABA). ② Soluble protein content was increased both in fresh weight and dry matter bases. ③ The novel proteins (14, 18.5, 25, 45 kDa) were induced by ABA and disappeared after plantlet regeneration, while proteins of the same molecular weight existed in intact mature seed embryos and disappeared when the seeds were germinating. ④ Moreover, the electrophoretic patterns of total soluble proteins from callus being cultured with ABA, and from callus with regenerating plantlets, were very similar to those of total soluble proteins from mature seed embryos and germinating seed embryos in SDS polyacrylamide gel plates. ⑤ The practice that the callus precultured with addition of ABA, especially 10 mgL^{-1} ABA, followed by transferring to a regeneration medium gave us a high frequency of plantlet regeneration observed in the long-term culture of callus.
Key words : Abscisic acid, Novel Proteins, *Oryza sativa*, Plant regeneration, Rice callus.

長期間培養したイネカルスの再分化と蛋白質新生に及ぼすアブシジン酸の影響 : 徐　正君・藤野介延・古谷ちひろ・喜久田嘉郎（北海道大学農学部）

要　旨 : 長期間継代培養したイネカルスの再分化と蛋白質新生に及ぼすアブシジン酸の影響について検討した．その結果は下記に要約される．1) ABA の添加によってカルスの見かけの生長量は低下したが，乾物重の増加は著しい．2) 乾物重当たりの可溶性蛋白質の量的増加が認められた．3) ABA は 14, 18.5, 25, 45 kDa の蛋白質を誘導するが，いずれの蛋白質分子も完熟種子胚に存在し，発芽にともなって消失する．4) ABA 添加培地に生育するイネカルスの可溶性蛋白質の電気泳動像（SDS-PAGE）は再分化カルスや，発芽種子胚の可溶性蛋白質の電気泳動と極めて類似している．5) 培地に 10 mgL^{-1} ABA を添加して培養すると 15 日後には再分化細胞集塊が形成され極めて高い頻度で不定芽形成を観察した．
キーワード : アブシジン酸，イネカルス，個体再生，*Oryza sativa*，蛋白質新生．

Abscisic acid (ABA) was found to be effective for promoting the frequency of normal somatic embryo development in cultured caraway cells maintained in the dark[1]. In rice callus as well as in other plant calli, the callus cells after the long-termed and repeated subcultures were losing their embryogenic potentials. Inoue and Maeda showed that the addition of ABA in the preculture medium, then kinetin in the later culture medium, stimulated the shoot-bud and plantlet formation in rice callus cultures, suggesting that ABA was effective during the preceding stage of organ formation and kinetin in the later stage of growth and development[9]. On the other hand, the growth of callus was remarkably inhibited by the addition of ABA in preculture medium[8,20].

However, these results are only a few observations, which can not reflect various physiological changes in cells due to ABA. In germinating seed embryos, the novel proteins were induced by the application of ABA and they were the same as those which appeared in intact mature seeds[16]. Furthermore, Higuchi and Maeda demonstrated that the growth of rice callus was inhibited by ABA added to the culture, and observed two kinds of external structures of callus culture by a scanning electron microscope (SEM). An attempt has been made to observe the changes of external and internal structures of callus and to relate the changes of isolated protein components. It is also important to investigate the cause and mechanism of the ABA effect.

The present report describes the effects of ABA on protein accumulation, novel protein induction and on the process of embryogenesis and/or organogenesis in the long-term culture of rice callus.

Table 1. Effect of ABA on growth and protein accumulation in a long-term cultured rice callus.

ABA addition (mgL^{-1})	Growth of callus mass		Total soluble protein
	Fresh weight (g)	Dry matter (g)	mgg^{-1} callus (F. wt)
1	13.834	1.079	8.592
2	10.644	1.023	11.920
4	9.474	1.002	13.653
6	8.519	0.950	14.873
8	7.980	0.923	15.630
10	7.317	0.887	18.425
12	6.950	0.875	20.121

Data were collected after 28 days culture, One gram fresh weight and/or 0.078 g dry weight of initial inoculum were subtracted from total weight.

Materials and methods

1. Callus initiation

The mature seeds of rice (*Oryza sativa* L. var. YUHKARA) were husked and surface sterilized with 70% alcohol for 1 min, followed by 0.5% sodium hypochlorite for 60 min, and then rinsed three times with sterilized water. The seeds were placed on a plate containing 20 ml modified MS-medium (MS-minerals[17] and vitamins[13]) supplemented with 4.0 mg L^{-1} 2,4-D (2,4-dichlorophenoxyacetic acid), 30 gL^{-1} sucrose, 2.0 g L^{-1} Casaminoacid (Difco) and 6 g L^{-1} agarose. The medium was adjusted to pH 5.8 with 0.1 M NaOH and heated at 121°C for 10 min. The culture were maintained in darkness at 25±1°C for callus formation.

2. Preculture conditions for plantlet regeneration from callus

The callus originated from scutellum was subcultured on our MS medium containing 2.0 mg L^{-1} 2,4-D, 30 g L^{-1} sucrose, 1.0 g L^{-1} Casaminoacid and 6 g L^{-1} agarose. Every 30 days, the callus was transferred to a fresh medium. The subcultures were repeated 6 times. Afterwards, the calli were divided into two groups for precultures. One group was transferred to the same fresh medium and cultured for another 28 days, and the other was transferred to a fresh medium supplemented with 2, 4, 6, 8, 10 and 12 mg L^{-1} ABA. These precultures were maintained in the dark at 25±1°C for 28 days.

After 28 days, the calli were transferred to a regeneration medium containing 5 mg L^{-1} kinetin, 0.05 mg L^{-1} 2,4-D, 60 g L^{-1} sucrose and 6 g L^{-1} agarose for plantlet regeneration. The cultures were maintained under a 16hr photoperiod at 37.5 μ mole m^{-2} s^{-1} photon density by Toshiba Plant Lux Fluorescent Tubes at 25±1°C.

3. Analysis of protein

Precultured callus on the 21 st day was blotted dry on filter papers, weighed and frozen at −85°C. The frozen callus was macerated in a chilled mortar with a pestle, added to 1 ml of 100 mM Tris buffer (pH 7.4) containing 10 mM marcaptethanol per gram cells, and homogenized with a glass homogenizer. The homogenate was centrifuged at 20,000× g for 10 min. The supernatant was collected in ultracentrifuge tubes and spun at 100,000×g for 15 min. The supernatant was saved for protein analysis by SDS-PAGE[14]. Total protein was measured according to the Bradford method[2].

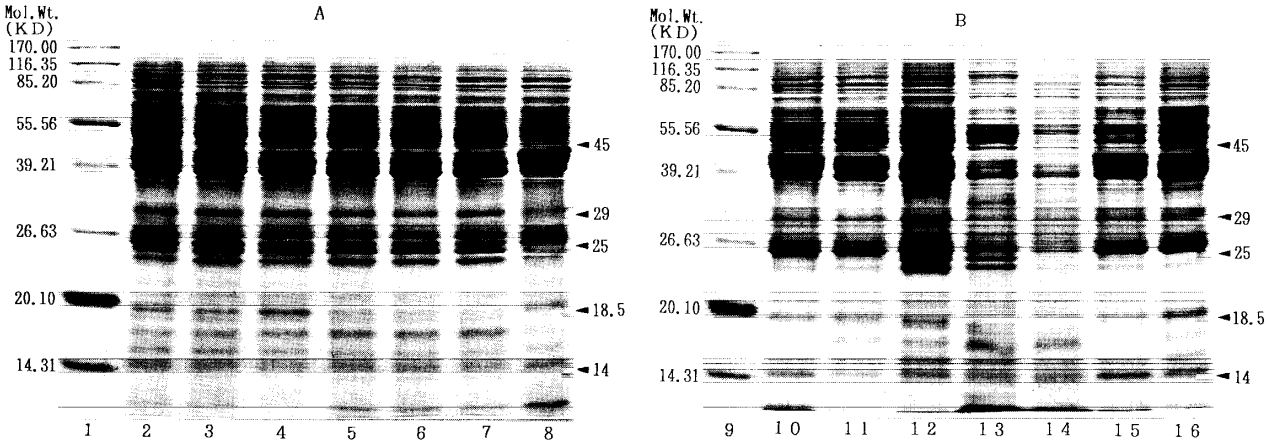
4. Histological observation

On the 21 st day, callus was fixed by FAA, dried with ethanol and embedded in paraffin block. Internal structures of cultures were observed by paraffin section stained with safranin and fast green.

Results

At first, ABA significantly inhibited callus growth on the basis of fresh weight, especially at a high concentration (6～12 mg L^{-1} ABA), but the decrease in matter was not significant in comparison to the control, as shown in Table 1. Secondly, ABA supplement was remarkably effective in increase in total soluble protein in cultured callus.

We have analysed the total soluble protein



A

lane 1 : Protein molecular weight marker.
lane 2 : Callus cultured with 2.0 mg L^{-1} ABA addition.
lane 3 : Callus cultured with 4.0 mg L^{-1} ABA addition.
lane 4 : Callus cultured with 6.0 mg L^{-1} ABA addition.
lane 5 : Callus cultured with 8.0 mg L^{-1} ABA addition.
lane 6 : Callus cultured with 10.0 mg L^{-1} ABA addition.
lane 7 : Callus cultured with 12.0 mg L^{-1} ABA addition.
lane 8 : Callus cultured without ABA addition.

B

lane 9 : Protein molecular weight marker.
lane 10 : Control callus without ABA addition.
lane 11 : Selected white-callus without ABA addition.
lane 12 : Callus cultured with 10.0 mg L^{-1} ABA addition.
lane 13 : Intact seed embryos.
lane 14 : Seed germinating.
lane 15 : Regenerants from callus precultured with 10.0 mg L^{-1} ABA addition.
lane 16 : Green spots from callus precultured with 10.0 mg L^{-1} ABA addition.

Fig. 1. SDS-PAGE separation of total soluble proteins isolated from ABA treated callus, regenerating callus and germinating seed embryos.

of callus cultured with additions of ABA and of regenerating callus through ABA preculture, then compared with the soluble protein of intact mature seed embryos and of germinating seed embryos without any ABA addition.

As shown in Fig. 1-A, in precultures with 2.0 mg L^{-1} ABA addition, 18.5, 25, 45 kDa novel proteins were induced (lane 3), and in high concentration (4.0～12 mg L^{-1} ABA) 14 kDa novel protein was additionally induced, while 29 kDa protein disappeared (lane 4～8). All of these proteins, synthesized *de novo* with the same molecular weight, were also presented in mature seed embryos (lane 13). The SDS-PAGE pattern of soluble proteins in the cases of 6, 8, 10, 12 mg L^{-1} ABA precultures (lane 4, 5, 6, 7) were especially similar to those of mature seed embryos (lane 13). The pattern of the selected white-callus cultures without ABA addition and the callus precultured with 2.0 mg L^{-1} ABA were similar, but not 29 kDa soluble protein, which was not found in the selected white-callus. Nevertheless, in the case of regenerated plantlets, the protein patterns (lane 15) were similar to that for germinating seed embryos (lane 14). Those novel proteins disappeared with regeneraton of plantlets, but not 45 kDa protein (lane 14 and 15 in Fig. 1 B). The internal structures of callus are shown in Fig. 2-A in that in the circumference of callus without ABA addition, the dividing cells were sporadic. However, in cultures with ABA addition, the cluster of dividing cells (meristematic cells) has been clearly observed (Fig. 2-B).

We have obtained high frequency plantlet regeneration from long-term subcultured calli through 28 days of ABA precultures. As shown in Table 2, with 6.0～12.0 mg L^{-1} ABA pretreatment, especially, 10.0 mg L^{-1} ABA addition to cultures, a long-term subcultured callus has expressed high frequency of plantlet regeneration in comparison with the control.

Table 2. Effect of ABA pretreatment on plantlet regeneration ftom a long-term cultured rice callus.

ABA pretreatment (mgL^{-1})	Plantlet regeneration		
	% of green plantlet[a]	Shoots per callus-mass[b]	Days after transfer to green plantlet
0 (control)	13.8%	2.78	18
2	11.7	3.10	20
4	15.2	2.67	17
6	18.4	3.47	15
8	26.6	3.87	15
10	37.8	5.10	11
12	28.7	5.33	11

Data were collected after 45 days.

$$a=\frac{\text{Number of callus mass with plantlets}}{\text{Total callus mass transplanted}}\times 100$$

$$b=\frac{\text{Total number of plantlets regenerated}}{\text{Number of callus mass with plantlets}}\times 100$$

But the effect of ABA preteatment on plantlet regeneration were not remarkable in the case of 2.0～4.0 mg L^{-1} ABA.

On the other hand, with ABA addition to preculture, the period for plantlet regeneration was shortened, especially at 10 mg L^{-1} ABA (Table 2) and was the optimal concentration to plantlet regeneration.

Discussion

In this study, we demonstrated the requirement of ABA for growth and plant regeneration from a long-term cultured rice callus initiated from mature seed, and we have also shown the effects of ABA on tissue structure, soluble protein content and protein component of rice callus. The effects of ABA on callus growth and plantlet regeneration were shown in rice anther culture[20] and seed callus culture[8]. Our investigations in Table 1 and Table 2 support these reports. However, the stimulative effect of ABA on plant regeneration also shows that the increase in callus mass with plant regeneration and the time required for plant regeneration are anticipatory (Table 2).

We have not obtained any somatic embryos from ABA treated callus by paraffin section, but found a kind of indication on characteristic structure of embryogeny (Fig. 2). As for histology, in general, the embryogenesis begins to start with one or a few cells. Karlson and Vasil reported the progress in embryogenic

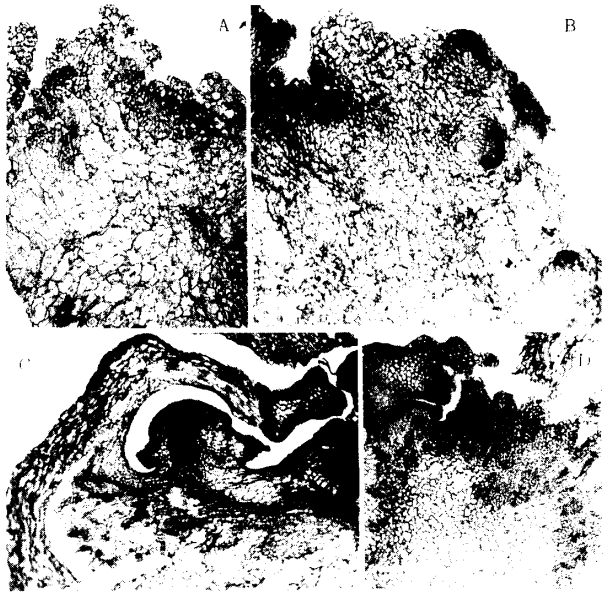


Fig. 2. Microphotographs of paraffin sections on various callus cultures.
A : Control callus culture without ABA addition (×50),
B : Callus cultured with 10.0 mg L^{-1} ABA addition (×50),
C : Somatic embryo in callus precultured with 10.0 mg L^{-1} ABA addition (×50),
D : Plantlet initial in callus precultured with 10.0 mg L^{-1} ABA addition (×50).

cell suspension cultures of *Panicum maximum* and *Pennisetum purpureum*[13]. Kamada and Harada stated the possible role of endogenous ABA on seed embryo formation[11]. On the

other hand, exogenous application of ABA stimulated complete embryonic development in caraway cultured cells[1]. According to the present results, we suggest that ABA promotes the progress of embryogenesis and/or organogenesis from cells of ordinary callus to embryogenic cells leading to the formation of prophase structure of embryogenesis and/or organogenesis, but can not develop perfect somatic embryos.

Furthermore, our experimental results indicate that the novel soluble proteins of 45, 25, 18.5 and 14 kDa were shown as ABA-inducible proteins (Fig. 1) in callus cultured with ABA addition. Mundy and Chua have reported 45, 39, 25, 23, 21 and 20 kDa ABA-inducible proteins in germinating seeds of rice and ABA 21 protein accumulation in suspension cells of rice[16]. However, are they the same proteins having the molecular weight from intact seed embryos and the ABA-inducible proteins from germinating seeds or the ABA-inducible proteins from cultured callus? It is not clear.

Several workers have indicated that endogenous ABA levels increase in plant tissues subjected to water stress by high osmoticum, NaCl, or drying[7,10,19]. Developmental studies have indicated that ABA induces the accumulation of specific mRNAs and protein late in embryogenesis in the seeds of diverse species[5,6,15]. While an ABA-inducible protein disappeared from the seeds of water-imbibing and germinating state[18]. According to these results, the causes of endogenous ABA are mainly plant adaptation of environmental stress by optimal conditions. Exogenous ABA causes the precocious accumulation of these mRNAs in immature embryos and their reappearance in germinating seeds[5]. Some of them are storage proteins[3,5] or enzyme inhibitors[15]. Exogenous ABA was reported to induce the formation of white and opaque callus containing starch grains in maize[4] and in rice culture[20]. In rice callus cultures, some of these effects are confirmed that ABA is responsible to the total soluble protein accumulation, and that parts of ABA-inducible proteins are the storage proteins because these novel proteins were completely disappeared except 45 kDa protein when plants were regenerated (Fig. 2). Although ABA also induced the formation of white callus in rice culture[8], the physiological characteristics of these induced white calli were unclear.

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References

1. Ammirato, P.V. 1977. Hormonal control of somatic embryo development from cultured cells of caraway. Plant Physiol. 59 : 579—586.
2. Bradford, M.M. 1976. A rapid and sensitive method for the quantification of microgram quantities of proteins utilizing the principle of protein-dye bonding. Anal. Biochem. 72 : 248—254.
3. Bray, E.A. and R.N.Beachy 1985. Regulation by ABA of β-conglycinin expression in cultured developing soybean cotyledons. Plant Physiol. 79 : 746—750.
4. Close, K.R. and L.A.Ludeman 1987. The effect of auxin-like plant growth regulators and osmotic regulation on induction of somatic embryogenesis from elite maize inbreds. Plant Sci. 52 : 81—89.
5. Finkelstein, R.R., K.M.Tenbarge, J.E.Shumway, and M.L.Crouch 1985. Role of ABA in maturation of rapeseed embryos. Plant Physiol. 78 : 630—636.
6. Galau, G.A., D.W.Hughes, and L.Dure 1986. Abscisic acid induction of cloned cotton late embryogenesis-abundant (Lea) mRNAs. Plant Mol. Biol. 7 : 155—170.
7. Henson, I.E. 1984. Effect of atmospheric humidity on abscisic acid accumulation and water stress in leaves of rice (*Oryza sativa* L.). Ann. Bot. 54 : 569—582.
8. Higuchi, N. and E.Maeda 1990. Enhanced plant regeneration in rice callus cultures following abscisic acid accumulation and water stress in leaves of rice (*Oryza sativa* L.). Ann. Bot. 54 : 569—582.
9. Inoue, M. and E.Maeda 1981. Stimulation of shoot bud and plantlet formation in rice callus cultures by two-step culture method using abscisic and kinetin. Jpn. J. Crop Sci. 50 : 381—322.
10. Jones, H., R.A.Leigh, A.D.Tomos and R.G.W. Jones 1987. The effect of abscisic acid on cell turgor pressures, solute content and growth of wheat roots. Planta 170 : 257—262.
11. Kamada, H. and H.Harada1992. Molecular regulation of somatic embryogenesis. Protein Nucleic Acid Enzyme 37 : 261—269*.
12. Karlsson, S.B. and I.K.Vasil 1986. Morphology

and ultrastructure of embryogenic cell suspension cultures of *panicum maximum* (Guinea grass) and *Pennisetum purpureum* (Napier grass). Am. J. Bot. 73 : 894—901.
13. Kikuta, Y. and Y.Okazawa 1984. Control of root and shoot-bud formation from potato tuber tissue cultured *in vitro*. Physiol. Plant. 61 : 8—12.
14. Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature 227 : 680—685.
15. Mundy, J., J.Hejgaard, A.Hansen, L.Hallgren, K. G.Jorgensen and L.Munck 1986. Differential synthesis *in vitro* of barley aleurone and starchy endosperm proteins. Plant Physiol. 81 : 630—636.
16. Mundy, J. and N.H.Chua 1988. Abscisic acid and water-stress induce the expression of a novel rice gene. EMBO J. 7 : 2279—2286.
17. Murashige, T. and F.Skoog 1962. A revised medium for rapid growth and bioassays with tabacco tissue cultures. Physiol. Plant. 15 : 473—479.
18. Shinozaki, K. and K.Y. Shinozaki 1992. Plant genes induced by drought stress and ABA (in Japanese). Protein Nucleic Acid Enzyme 37 : 194—203*.
19. Skriver, K. and J.Mundy 1990. Gene expression in response to abscisic acid and osmotic stress. Plant Cell 2 : 503—512.
20. Torrizo, L.B. and F.J.Zapata 1986. Anther culturre in rice. IV. The effect of abscisic acid on plant regeneration. Plant Cell Rep. 5 : 136—139.
21. Vasil, V. and I.K.Vasil 1982. Characterization of an embryogenic cell suspension culture derived from cultured influorescences of *Pennisetum americanum* (Pearl milet, Gramineae). Am. J. Bot. 69 : 1441—1449.

* In Japanese.