Survival mechanism of dried calli and regeneration of plants in rice

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(Received 22 September 1990)

ABSTRACT

A system for long-term dry preservation of calli of rice (*Oryza sativa* L.) and a protection mechanism regulating the survival of dried calli were investigated. The highest survival of dried calli and the highest regeneration rate of plantlets were observed in calli which had been pretreated with 10^{-5} M abscisic acid (ABA) in the presence of 90 g/l of sucrose and were regrown on an R-2 medium. We detected a corresponding accumulation of the transcript RNA of the *rab 16A* gene (a rice gene induced by ABA and water stress) in dried calli, mature seeds, and calli pretreated with 10^{-5} M ABA. The levels of this mRNA increased with the increase of the sucrose concentration, indicating that the accumulation of *rab 16A* mRNA is regulated by ABA at higher concentrations of sucrose and related to the survival of dried calli. Analysis of proteins by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) demonstrated similar protein patterns in fresh and dried calli. However, in the dried calli pretreated with 10^{-5} M ABA and 90 g/l of sucrose, different protein patterns were found compared to those in a callus dried without the pretreatment, indicating that some specific polypeptides might be synthesized in the pretreated dried callus.

1. INTRODUCTION

Recently, it has been recognized that higher plants can respond to various environmental changes by inducing the formation of specific mRNAs and proteins such as heat shock proteins, pathogenesis-related proteins, etc. Drying or water stress allows the plant to adapt itself and to be protected from other stress conditions. Such a protection mechanism seems to be programmed in embryo cells. If we could regulate the mechanism in dedifferentiated cells, the callus tissue could stably be preserved in a dried state for a long period of time. Survival of

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dried carrot calli pretreated with abscisic acid (ABA) at a high sucrose concentration was observed for one year after drying (Nitzsche, 1978, 1980). In addition, a callus which is a dedifferentiated tissue uses some different systems of gene regulation such as the occurrence of a high frequency of somatic mutations observed among the regenerated plants (Oono, 1981), DNA amplification and diminution (Kikuchi et al., 1987), and heritable gene inactivation (Oono, 1985). Therefore, we assume that some of the genes which are related to the protection mechanisms of cells from drying may be activated or induced in a callus.

ABA is a plant growth regulator known to accumulate in higher plants in response to drought stress. ABA induced the accumulation of specific mRNAs and proteins during the process of seed maturation in several species and some of the late developmental mRNA in cereal seeds is rapidly degraded during seed germination (Finkelstein et al., 1985; Mundy et al., 1986; Galau et al., 1986; Litts et al., 1987). However, exogenous application of ABA results in the precocious accumulation of these mRNAs in immature embryos and their reappearance in germinating seeds, as well as in the stimulation of synthesis of embryo-specific storage proteins in embryos (Finkelstein et al., 1985; Mundy et al., 1986; Crouch et al., 1981). ABA-inducible proteins may be involved in seed protection and/or the maintenance of dormancy. In physiological studies, it was reported that endogenous ABA levels in plant tissues increased when the tissues were subjected to water-stress induced by a high osmoticum, NaCl, or drying (Henson, 1984; Jones et al., 1987). The specific mRNAs and proteins accumulated under these conditions could affect intra-cellular osmolality or confer other protective functions (Finkelstein et al., 1986; Ramagopal, 1987). A novel rice gene *rab 16A* (stands for Responsive to ABA), which is induced when rice plants are subjected to water-stress, was identified by Shinozaki et al. (1989). *rab 16A* mRNA and protein accumulated in rice embryos, leaves, roots, and callus-derived suspension cells when the tissues were treated with NaCl and/or ABA. We are interested in developing a system for long-term preservation of the rice callus in a dried state, and are investigating the molecular mechanism responsible for the survival of the dried rice callus, for the purpose of eventually achieving long-term dry preservation of calli or plant cells and plant regeneration during preservation.

2. MATERIALS AND METHODS

Callus culture

Rice cultivar Norin 8 (N8P3), Nipponbare which was harvested in the experimental field of the National Institute of Agrobiological Resources, Japan, was used in this experiment. Dehulled mature seeds of rice were sterilized with 70% ethanol for 1 minute followed by 1% sodium hypochlorite solution for 15 minutes and then washed several times with sterile water. A callus was induced in an illuminated room at $25 \pm 1^\circ\text{C}$ from mature seeds on Murashige Skoog (MS)

(Murashige et al., 1962) basal medium supplemented with 2.0 mg/l of 2,4-D (2,4-dichlorophenoxy acetic acid). The callus was subcultured in the same MS medium. The shoot regeneration medium consisted of the MS medium supplemented with 10^{-7} M NAA (α -naphthalene acetic acid), 10^{-6} M kinetin, 35 g/l of sorbitol, 3 g/l of casein hydrolysate. Shoots were regenerated in the light at $25 \pm 1^\circ\text{C}$.

Pretreatment with ABA and sucrose

Six week-old callus pieces (about 50 mg each) were transferred into MS medium supplemented with 10^{-5} M ABA at 30, 50, 70, and 90 g/l of sucrose, respectively, and incubated at $25 \pm 1^\circ\text{C}$ for 2 days.

Callus drying and survival of dried calli

Calli were dried on sterile petri dishes under the air stream of a laminar flow cabinet for 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 12, 24, 48, 72, and 120 hours (h) at room temperature (normal drying) and in sterile petri dishes containing MS medium for 3 days (slow drying). The calli dried for 7 h under the normal drying conditions and 3 days under the slow drying conditions were preserved in 10 ml-glass ampoules. R-2 medium containing 0.5 mg/l of 2,4-D with or without 2 mg/l of gibberellic acid (GA) and 0.2 mg/l of 6-benzylamino purine (BA) (Ohira et al., 1973) and MS medium containing 2.0 mg/l of 2,4-D with 1, 10, 50, and 100 mg/l of ascorbic acid were used for the regrowth of the dried callus. For the survival of the dried callus, more than 50 callus pieces were inoculated on the regrowth medium. The survival frequency based on the resumption of proliferation was determined 20 days after inoculation, and was expressed as;

$$\% \text{ survival} = \frac{\text{Number of calli which survived}}{\text{Total number of calli inoculated}} \times 100$$

Total RNA isolation

Total RNAs from the calli, shoots and mature seeds were prepared. A callus sample (2 g) was frozen in liquid nitrogen, ground into powder in a mortar and homogenized in 7.5 ml of extraction buffer [50 mM Tris-HCl (pH 8.0), 300 mM NaCl, 5 mM EDTA (pH 8.0), 2% SDS, 2 mM aurintricarboxylic acid, 14 mM 2-mercaptoethanol] with a pestle for several minutes. The homogenate was centrifuged at 8,000 rpm for 5 minutes at 4°C . The aqueous phase was passed through one layer of nylon mesh (680 μm) and precipitated overnight in a cold room after addition of 0.33 vol of 8 M LiCl. The precipitate was collected by centrifugation and then resuspended in 2 ml of sterile water. An equal volume of phenol was added to the extract which was shaken for several minutes at room temperature. The aqueous phase was collected and precipitated overnight at -20°C after addition of 0.1 vol of 5 M NaCl and 2.5 vol of ethanol. The RNA

precipitate was collected by centrifugation, washed with 70% ethanol and dissolved in sterile water.

RNA blot analysis

Total RNAs were separated by electrophoresis in 1.2% agarose gels, transferred onto nitrocellulose filter and hybridized with purified *NheI*-fragment DNA from the ABA-inducible *rab 16A* gene of rice by the procedure of Maniatis et al. (1982). The filter was incubated at 42°C overnight in 25 mM NaPO₄ (pH 6.7) buffer containing 50% formamide, 5×SSC, 10×Denhart's solution and 250 µg/ml of denatured salmon sperm DNA. Hybridization was performed by the addition of ³²P-labeled probe DNA at 42°C for 24 h.

Protein analysis

For the isolation of proteins a callus sample was ground in a mortar and extracted in two vol. of lysis buffer [8.5 M urea, 2% triton X100, 1.6% ampholine (pH 5–7), 0.4% ampholine (pH 3.5–10.0), 5% 2-mercaptoethanol and 5% polyvinylpyrrolidone K30]. The extracted proteins were loaded onto isoelectric focusing (IEF) rod gels. IEF and two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) were performed by a modified procedure of the O'Farrell system (1975) with 12.5% polyacrylamide in the separation gel and 4% in the stacking gel. After electrophoresis, the gel was stained with silver for the detection of the protein spots.

3. RESULTS

Drying of callus

Fifty milligram pieces of six week-old callus sample were dried in a laminar air-flow cabinet at room temperature, and in MS medium in a laminar air-flow cabinet for the slow drying. During the drying process in the laminar air-flow cabinet, a marked decrease in the water content of the callus was observed until 4 h after drying, and a smaller decrease after 7 h drying, the total decrease amounting to 90% of the callus weight (Fig. 1). Three days were required for the drying of the callus subjected to slow drying. Water losses of the calli pretreated with ABA at different concentrations of sucrose decreased in the medium containing higher concentrations of sucrose (Fig. 2).

Survival of dried callus and plant regeneration

In order to identify the factors affecting the survival of the dried callus, the callus pieces were treated with 10⁻⁵ M ABA and 200 mM NaCl at different concentrations of sucrose for 2 days prior to drying. Two media with a different composition of inorganic salts were used for testing the survival of the dried calli. The callus survival frequency was different depending upon the pretreatments

Fig. 1. Decrease in callus weight during drying in a laminar air-flow cabinet at room temperature.

Fig. 2. Water loss in calli dried for 7 h in a laminar air-flow cabinet when treated with 10^{-5} M ABA at different concentrations of sucrose.

and the regrowth media, ranging from 0 to 80% when the callus was preserved for 5 days after drying (Tables 1 and 2). Calli pretreated with a higher concentration of sucrose and NaCl did not survive regardless of the type of regrowth medium was used. However, pretreatment with 10^{-5} M ABA at higher concentrations of sucrose resulted in the survival of the dried callus. The highest survival rate of dried calli was observed when the calli were pretreated with 10^{-5} M ABA at 90

Table 1. Effect of 2-day pretreatment with ABA and NaCl at different sucrose concentrations on survival of dried callus in R-2 medium (Data indicate the percentage of survival¹⁾)

Pretreatment	Normal drying		Slow drying (90 DOP)
	5 DOP ²⁾	90 DOP ²⁾	
<i>Sucrose (g/l)</i>			
50	0	0	0
70	0	0	16.7
90	0	0	0
<i>NaCl (200 mM)+ sucrose (30 g/l)</i>	0	0	0
<i>ABA + Sucrose</i>			
10 ⁻⁵ M+ 70 g/l	50.0 b	0	87.5 a
10 ⁻⁵ M+ 90 g/l	83.3 a	0	79.2 a
10 ⁻⁵ M+110 g/l	12.5 c	0	33.3 b

¹⁾ In a column, means followed by a common letter are not significantly different at the 5% level by DMRT. The calli dried for 7 h (normal drying) and 3 days (slow drying) were preserved in ampoules.

²⁾ DOP: days of preservation.

Table 2. Survival of dried rice calli pretreated with ABA and sucrose on different regrowth media¹⁾

Pretreatment: ABA + Sucrose	R-2 medium				MS medium ²⁾				
	—	GA	BA	GA + BA	—	A1	A10	A50	A100
10 ⁻⁵ M + 70 g/l	50.0	79.2	45.3	45.8	29.2	29.2	33.3	54.2	33.3
10 ⁻⁵ M + 90 g/l	38.3	75.0	58.3	62.5	50.0	54.2	70.8	58.3	62.5

¹⁾ The calli pretreated with ABA sucrose, normally dried for 7 h, and preserved for 5 days were used.

²⁾ A1, A10, A50, A100 = 1, 10, 50, 100 mg/l of ascorbic acid, respectively.

g/l of sucrose, preserved for 3 months, and regrown on R-2 medium supplemented with 0.5 mg/l of 2,4-D and 30 g/l of sucrose (Table 1). The survival frequency of the dried callus in R-2 medium was higher than that in MS medium. There were also some effects of GA, BA, and ascorbic acid on the survival of the dried callus, although it was difficult to obtain consistent results in these treatments (Table 2).

The surviving calli recovered from dry preservation were transferred into the regeneration medium. Plantlets were regenerated with a frequency similar to that of an intact fresh callus (ca. 60%), indicating the possibility of dry preservation of the rice callus without loss of totipotency (Fig. 3).

Fig. 3. Survival of the dried callus and regeneration of plants from calli : calli pretreated with 10^{-5} M ABA and 90 g/l of sucrose and slowly dried for 3 days (A), dried calli preserved in ampoules (B), surviving calli in R-2 medium (C), regenerated plantlets (D).

Accumulation of rab 16A mRNA induced by drying and pretreatment with ABA and sucrose

A Northern blot analysis was employed to analyze the levels of *rab 16A* mRNA induced by water stress in various rice tissues which were dried or pretreated with ABA and sucrose. The increased level of ABA in the plant tissues subjected to water stress or drying induces an accumulation of specific mRNAs and proteins which may confer a protective function (Finkelstein and Crouch, 1986; Ramagopal, 1987). During normal callus drying, there was an accumulation of *rab 16A* mRNA and the levels of *rab 16A* mRNA increased when the time of callus drying was prolonged (Fig. 4). High levels of mRNA accumulated in calli

Fig. 4. Northern hybridization of *rab 16A* with total RNAs of calli normally dried for 7 h (lanes 1–9), mature seed (lane 10), 10^{-5} M ABA treated callus (lane 11), and fresh callus (lane 12).

dried for more than 8 h and in mature seeds as well. The *rab 16A* mRNA was not detected in fresh shoots and roots which were grown for 10 days in a greenhouse. However, mRNA accumulated in dried shoots (data not shown).

In the treatment with ABA, *rab 16A* mRNA accumulation was detected although the levels of mRNA increased with the increase of the sucrose concentration (Fig. 5). This finding suggests that the accumulation of *rab 16A* mRNA is regulated by ABA at higher concentrations of sucrose. The survival of the dried calli was the highest in those pretreated with 10^{-5} M ABA at 90 g/l of sucrose and slowly dried for 3 days (Table 1). In this study, it is difficult to conclude that the accumulation of *rab 16A* mRNA resulted in survival of the dried calli because mRNA accumulated by drying in the absence of ABA and sucrose treatment.

Fig. 5. Northern hybridization of *rab 16A* with the total RNAs of fresh calli subjected or not subjected to treatment with 10^{-5} M ABA at different sucrose concentrations.

Fig. 6. Protein patterns separated by two-dimensional gel electrophoresis in a fresh callus (A), dried callus (normal drying for 7h) (B), dried callus with pretreatment with 10^{-5} M ABA and 90 g/l of sucrose (slow drying) (C), a surviving callus in the regrowth medium followed by pretreatment and slow drying for 3 days (D). The size markers are as follows : α -lactalbumin (14.4 kDa), soybean trypsin inhibitor (20.1 kDa), carbonic anhydrase (30 kDa), ovalbumin (43 kDa), bovine serum albumin (67 kDa), and phosphorylase b (94 kDa). The spots indicated by solid arrows show an increased intensity, and those by open triangles, a decreased intensity.

Protein patterns of dried and ABA-treated calli

The patterns obtained by 2D-PAGE for proteins extracted from the calli subjected or not subjected to the pretreatment with 10^{-5} M ABA in the presence of 90 g/l of sucrose and dried under normal and slow drying conditions are shown in Fig. 6. The analysis was restricted to a few prevalent spots which showed changes in the relative intensity after callus drying. Analysis of the proteins by 2D-PAGE revealed similar patterns for the proteins extracted from the fresh and dried calli without pretreatment with ABA and sucrose. However, in the slowly dried calli pretreated with 10^{-5} M ABA and 90 g/l of sucrose, different patterns of proteins were found compared to those of the fresh and dried calli without the pretreatment. There were new spots indicated by solid arrows particularly in the 14 to 20 kD region or spots with an increased intensity after callus drying followed by the pretreatment. The gene *rab 16A* induced by ABA and water stress is a member of a small multigene family which encodes proteins with Mr 15500–16800, suggesting that the corresponding polypeptides account for an increased proportion of the proteins newly synthesized during callus drying. In contrast, in the spot indicated by an open triangle the intensity of the polypeptides decreased after callus drying. In addition, the newly found spots in the ABA-treated dried calli disappeared or their intensity decreased in the surviving calli which were incubated in the regrowth medium for 2 days followed by the pretreatment and drying.

4. DISCUSSION

Callus or plant cell preservation has become important to secure long-term storage of germplasm and to prevent changes in morphogenetic potential, production of useful chemicals, and genetic characters caused by repeated subculturing. It is therefore essential to investigate the factors and protection mechanisms regulating the survived processes of dried calli, which may be useful for long-term preservation of a callus. Dried calli of carrot pretreated with ABA and a high concentration of sucrose have been stored for 1 year (Nitzsche, 1980), and plantlets were regenerated from the callus which survived after drying (Kaimori et al., 1989). We are interested in investigating the survival of the dried callus and survival mechanisms in rice which is a monocot.

Survival of the dried callus was investigated by subjecting it to pretreatment with ABA and NaCl at different sucrose concentrations. We observed that pretreatments with 10^{-5} M ABA in the presence of high concentrations of sucrose were effective in the survival of the dried callus, indicating that ABA may play an important role in the survival of dried callus. In plant tissues, the increase in the level of ABA by drying leads to the accumulation of specific mRNAs and proteins which may confer protective functions. The difference in the survival frequencies

depended on the regrowth media used and R-2 medium gave a higher rate of survival than MS medium. Properties of cultured cells such as growth rate and formation of aggregates are known to be influenced by the nutritional conditions of the culture medium (Murashige et al., 1963). In the R-2 medium the concentrations of nitrogen, sucrose, and 2,4-D were lower than those of the MS medium.

In addition, there was a great difference in the survival of the dried callus between the drying methods. The viability of a callus dried in MS medium for slow drying was maintained for 3 months after drying, while that of a callus dried normally was lost, suggesting that some of the processes involved in the protection of a callus from drying may operate during slow drying. In this study, we developed a system for the long-term preservation of the rice callus by drying. The system consists of, 1) pretreatment with 10^{-5} M ABA at 90 g/l of sucrose, 2) slow drying in a medium, and 3) regrowth on R-2 medium. This dry callus preservation system was also effective in other Japonica rice varieties, Nipponbare and Waseshirage and Indica varieties, Azucena, Te-tep and Zenith (data not shown).

For the preservation of a callus, the maintenance of totipotency is the most important factor. We obtained plantlets regenerated from a callus which survived after dry preservation indicating the possibility of long-term dry preservation of a callus and/or a plant cell without loss of totipotency. Therefore, we are interested in investigating the molecular mechanisms of survival of the dried callus.

Northern blot hybridization showed that *rab 16A* mRNA accumulated in mature seeds, dried shoots and calli, indicating that the *rab 16A* gene expression is not tissue-specific. However, the sizes of the transcripts detected in the Northern blot analysis varied among the tissues. The transcripts of mature seeds and shoots (1.1 kb) were smaller than that of a callus (1.3 kb) suggesting that splicing may not be involved in the callus tissue and sizes were larger than that of *rab 16A*, 0.85 kb. Furthermore, *rab 16A* mRNA accumulated in the calli pretreated with ABA in the presence of higher concentrations of sucrose. The relationship between the accumulation of *rab 16A* mRNA and survival of the dried callus is illustrated by our observation that the accumulation of *rab 16A* mRNA was regulated by ABA in the presence of higher concentrations of sucrose at which the highest survival rate of the dried callus was observed. The reason for the difference in the sizes of the transcripts of *rab 16A* between tissues and between *rab 16A* and its transcript remains to be elucidated.

In the investigation on the protein patterns of fresh and dried calli with or without pretreatment with ABA and sucrose, we observed that the protein patterns changed in the dried callus followed by the pretreatment. However, the changes in the dried callus without the pretreatment were negligible compared to those of the fresh callus, indicating that the ABA and sucrose treatment induced the alteration of specific protein synthesis. Recently, it has been shown that the

increased levels of endogenous ABA in plants subjected to water stress by drying, high osmoticum, or NaCl induce the formation of specific mRNAs and proteins which confer protective functions against water stress (Finkelstein et al., 1986; Ramagopal, 1987). Our results indicate that the synthesis of a limited number of proteins is not altered by drying, but by exogenous ABA at higher concentrations of sucrose, although *rab 16A* mRNA accumulated in both treatments. This phenomenon may be due to the incomplete translation or absence of translation of the specific mRNAs of the gene(s) induced by drying, which may be responsible for the survival of the dried callus. This phenomenon also suggests that the gene(s) responsible for the protection of a cell from water stress or cell drying should be induced or activated prior to cell drying in order to secure the survival of dried plant cells.

It appears that cell culture is a prerequisite to progress in plant biotechnology. Mature seeds of cereal plants can be preserved for more than ten years under appropriate conditions. However, presently, it is difficult to preserve recalcitrant seed species or woody plants as genetic materials under dry conditions. It would be interesting to identify the mechanism whereby such plant species can tolerate drying. The present results indicate that a rice plant cell can be preserved under certain conditions for a long time. In our studies, we demonstrated that a callus, which is a dedifferentiated tissue, could be preserved by pretreatment with ABA in the presence of a higher concentration of sucrose, and that the gene(s) responsible for protection from dry preservation could be activated or induced by the pretreatment, indicating that the gene(s) can be manipulated and then introduced into plant cells.

We thank Mr. M. Fukuda for the excellent technical help in protein analysis, Drs. F. Takaiwa, S. Kikuchi, M. H. Soliman for helpful suggestions for this manuscript. Dr. D. H. Shin was awarded a STA (Science and Technology Agency, Japan) Fellowship, and Ms. S. Virigool, a MAFF (Ministry of Agriculture, Forestry, and Fisheries, Japan) Fellowship for this work.

REFERENCES

- BAJAJ, Y. P. S. and REINERT, T. (1977) Cryobiology of plant cell cultures and establishment of gene-banks. In: *Plant Cell, Tissue, and Organ Culture*, (ed: J. Reinert and Y. P. S. Bajaj), pp. 757-789. Springer-Verlag, Berlin.
- CROUCH, M. L. and SUSSEX, I. M. (1981) Development and storage-protein synthesis in *Brassica napus* L. embryos in vivo and in vitro. *Planta* **153**, 64-74.
- FINKELSTEIN, R. R. and CROUCH, M. L. (1986) Rapeseed embryo development in culture on high osmoticum is similar to that in seeds. *Plant Physiol.* **81**, 907-912.
- FINKELSTEIN, R. R., TENBARGE, K. M., SHUMWAY, J. E. and CROUCH, M. L. (1985) Role of ABA in maturation of rapeseed embryos. *Plant Physiol.* **78**, 630-636.
- GALAU, G. A., HUGHES, D. W. and DURE III, L. (1986) Absciscic acid induction of cloned cotton late embryogenesis abundant(Lea) mRNA. *Plant Mol. Biol.* **7**, 155-170.
- HENSON, I. E. (1984) Effects of atmospheric humidity on absciscic acid accumulation and water status in leaves of rice (*Oryza sativa* L.). *Ann. Bot.* **54**, 569-582.

- JONES, H., LEIGH, R. A., TOMOS, A. D. and JONES, R. G. W. (1987) The effect of abscisic acid on cell turgor pressures, solute content and growth of wheat roots. *Planta* **170**, 257–262.
- KAIMORI, N. and TAKAHASHI, N. (1989) Plant regeneration from dried callus of carrot (*Daucus carota* L.). *Japan. J. Breed.* **39**, 379–382.
- KIKUCHI, S., TAKAIWA, F. and OONO, K. (1987) Variable copy number DNA sequences in rice. *Mol. Gen. Genet.* **210**, 373–380.
- KING, R. W. (1976) Absciscic acid in developing wheat grains and its relationship to grain growth and maturation. *Planta* **132**, 43–51.
- LITTS, J. C., COLWELL, G. W., CHAKERIAN, R. L. and QUATRANO, R. S. (1987) The nucleotide sequence of a cDNA clone encoding the wheat Em protein. *Nucl. Acids Res.* **15**, 3607–3618.
- MANIATIS, T., FRITSCH, E. F. and SAMBROOK, J. (1982) *Molecular Cloning: A Laboratory Manual*, p. 545. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
- MUNDY, J., HEJGAARD, J., HANSEN, A., HALLGREN, L., JORGENSEN, K. G. and MUNCK, L. (1986) Differential synthesis in vitro of barley aleurone and starchy endosperm proteins. *Plant Physiol.* **81**, 630–636.
- MURASHIGE, T. and SKOOG, F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* **15**, 473–497.
- NITZSCHE, W. (1978) Erhaltung der Lebensfähigkeit in getrocknetem Kallus. *Z. Pflanzenphysiol.* **87**, 469–472.
- NITZSCHE, W. (1980) One year storage of dried carrot callus. *Z. Pflanzenphysiol.* **100**, 269–271.
- O'FARRELL, P. H. (1975) High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* **250**, 4007–4021.
- OHIRA, K., OJIMA, K. and FUJIWARA, A. (1973) Studies on the nutrition of rice cell culture I. A simple, defined medium for rapid growth in suspension culture. *Plant Cell Physiol.* **14**, 1113–1121.
- OONO, K. (1981) In vitro method applied to rice. In: *Plant Tissue Culture Methods and Applications in Agriculture*. (ed: T. A. Thorpe), pp. 273–298. Academic Press, New York.
- OONO, K. (1985) Putative homozygous mutations in regenerated plants of rice. *Mol. Gen. Genet.* **198**, 377–384.
- RAMAGOPAL, S. (1987) Differential mRNA Transcription during salinity stress in barley. *Proc. Natl. Acad. Sci. USA* **84**, 94–98.
- SINOZAKI, K.-Y., MUNDY, J. and CHUA, N. H. (1989) Four tightly linked *rab* genes are differentially expressed in rice. *Plant Mol. Biol.* **14**, 29–39.
- SUZUKI, Y., KUROGOCHI, S., MUROFUSHI, N., OTA, Y. and TAKAHASHI, N. (1981) Seasonal changes of GA₁, GA₁₉, and abscisic acid in three rice cultivars. *Plant Cell Physiol.* **22**, 1085–1093.