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Establishment of a system of high-frequency embryogenesis from long-term cell suspension cultures of rice (*Oryza sativa* L.)

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Summary. Suspension cultures which maintained embryogenic potency for more than 18 months were established from excised immature embryos of rice (*Oryza sativa* L. cv. Konansou). The cultures were subcultured every three days in N6 medium supplemented with proline (10 m*M*), casein hydrolysate (300 mg/l), sucrose (30 g/l) and 2,4-D (1 mg/l). The frequency of embryogenesis from the embryogenetic suspension cultures reached about 90% when cell clusters (about 1 mm in diameter) were transferred to a solid medium which consisted of N6 medium, NAA (1 mg/l), kinetin (5 mg/l), sucrose (30 g/l) and Gelrite (2 g/l). When smaller clusters of cells (approximately 200–400 μm in diameter) were transferred to a liquid medium which consisted of salts of N6 medium diluted with an equal volume of water plus sucrose (45 g/l), NAA (0.01 mg/l) and 4-PU (0.1 mg/l) at a cell density of 13 clusters/ml in 2 ml of medium, somatic embryogenesis was initated at high frequency (about 50%). Morphological evidence is provided to demonstrate that the regeneration occurred via embryogenesis. This is the first report of high-frequency embryogenesis in suspension cultures of rice cells.

Key words: Rice – Embryogenesis – Proline – Casein hydrolysate

Introduction

In recent years, there have been reports of successful in vitro somatic embryogenesis of many important graminaceous crops (Green 1982; Vasil and Vasil 1980, 1981 a, b; Wernicke and Brettell 1980). However, the frequency of regeneration has been low and cells have tended to lose the potential for embryogenesis within a short time during subculture (Bhattacharya and Sen 1980; Tamura 1968).

Regeneration of plants from tissue cultures of rice was first reported independently by several Japanese researchers (Kawata and Ishihara 1968; Maeda 1968; Niizeki and Oono 1968; Nishi et al. 1968; Tamura 1968). Recently, many researchers have reported somatic embryogenesis in rice (Raghava-Ram and Nabors 1985; Chen et al. 1985; Ling et al. 1983; Wernicke et al. 1981; Abe and Futsuhara 1985). However, in these reports, successful embryogenesis occurred rather sporadically and transiently in some cases. In most experiments, embryogenesis was induced on solid media from large clusters of cells. Success in the induction of embryogenesis in a liquid medium has been not reported.

It is important for physiological and biochemical investigations, as well as for mass propagation, to establish suspension cultures of embryogenic cells which can maintain high embryogenic potential for a long time and to induce somatic embryos at high frequency from small clusters of cells in a liquid medium. In this paper, we report the establishment of suspension cultures of rice cells which maintain a high potential for embryogenesis over a long period and we also report the induction of embryogenesis at high frequency in a liquid medium as well as on a solid medium.

Material and methods

Initiation and maintenance of calli

Thirteen cultivars of rice (*Oryza sativa* L.), 'Tiyonishiki', 'Miyazaki 25', 'Toyonishiki', 'Tou 108', 'Miyazaki 29', 'Kamenoo', 'Shindaiokoshi', 'G 88', 'G 11' (japonica rice), 'Konansou', 'Blue-

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belle', 'Jamuna' and 'IR 24' (indica rice), were grown in a greenhouse. Between 7 and 8 days after pollination, developing kernels were sterilized in 10% sodium hypochloride for 20 min. After three rinses with sterilized water, the immature embryos were dissected out from the kernels and placed on solid N6 medium (Chu et al. 1975) or on solid MS medium (Murashige and Skoog 1962), as modified by Inoue and Maeda (1980) (R-MS medium), supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) (1 mg/l), sucrose (30 g/l) and Gelrite (2 g/l). The media were adjusted to a pH of 5.8 before autoclaving. The cultures were incubated at 27 °C in the dark and were subcultured every 2 weeks in the same medium. Calli subcultered for more than 3 months were used in the experiments.

For establishment of suspension cultures, 3-month-old calli derived from immature embryos of the cultivar Konansou were used. The calli were transferred to 100-ml Erlenmeyer flasks that contained 20 ml N6 or R-MS liquid medium supplemented with 2,4-D (1 mg/l), sucrose (30 g/l), casein hydrolysate (0–300 mg/l) and L-proline (0–25 m*M*). The cells were transferred to fresh medium every 3 days. The cultures were incubated at 27 °C in the dark on a reciprocal shaker (80 strokes/min, with an amplitude of 5 cm).

Regeneration of plants

For regeneration on a solid medium, nine pieces of calli (1–1.5 mm in diameter) were transferred onto solid N6 medium supplemented with naphthaleneacetic acid (NAA) (1 mg/l), kinetin (5 mg/l), sucrose (3%) and Gelrite (2 g/l) in petri dishes (70 × 30 mm). The calli were incubated at 27 °C in the dark.

For regeneration in a liquid medium, the clusters of cells obtained by sieving calli through nylon screens with pore sizes from 198–423 μm were washed four times with N6 medium and transferred to N6 medium, diluted 1:1 with water (1/2 N6 medium), supplemented with different levels of indole-3-acetic acid (IAA), NAA, 2,4-D, kinetin and sucrose. The cells suspended in 2 ml of the medium were rotated horizontally in a tube (18 × 180 mm) at 15 rpm at 27 °C in the dark.

The method for calculation of the frequency of regeneration is described in the legends to the Tables. Experiments were repeated at least three times in each case.

Results

Selection of cultivars

The frequency of regeneration on solid medium varied with the varieties of rice examined (Table 1). The cultivars Konansou and Kamenoo showed higher frequencies of regeneration than other varieties. However, significant differences in the frequency of differentation were not observed between the subcultures grown on N6 and R-MS medium.

Establishment of suspension cultures with high potential for regeneration

We attempted to establish suspension cultures in which the potential for regeneration would be maintained for a long period of time, using calli initiated from the cultivar Konansou. Cells of these calli did not proliferate in liquid R-MS medium that contained 2,4-D (1 mg/l) and eventually died. However, the calli grew vigorously when subcultured in R-MS medium supplemented with casein hydrolysate (300 mg/l) or casein hydrolysate (300 mg/l) and L-proline (25 m*M*). When cells were subcultured every 3 days in these supplemented R-MS media and transferred to the solid differentiation medium, the frequency of regeneration reached about 50% (Table 2). By contrast, the calli grew vigorously N6 medium even in the absence of proline and/or casein hydrolysate. The frequency of regeneration was about 50% when cells were subcultured every 3 days in the liquid medium without proline and then transferred to the solid differentiation medium. The frequency of regeneration was increased markedly to about 90% by subculturing in N6 supplemented with L-proline, while the addition of casein hydrolysate increased the frequency of regeneration only slightly (Table 2).

The intervals between subcultures were another important factor affecting the frequency of regeneration. When cells were subcultured every 7 days, they did not regenerate at all, but the frequency of regeneration reached almost 90% if cells were subcultured every 3 days (Table 3).

Table 1. Differentiation in calli derived from immature embryos of various varieties of rice. Calli were subcultured every 14 days on R-MS or N6 medium that contained sucrose (30 g/l), 2,4-D (1 mg/l) and Gelrite (2 g/l). Calli (about 1 mm in diameter) were transferred to N6 medium that contained NAA (1 mg/l), kinetin (5 mg/l), sucrose (30 g/l) and Gelrite (2 g/l). Results were scored at the end of 14 days of culture on differentiation medium. Even if multiple embryos were induced on a single callus, it was regarded as a single differentiated callus

Variety	Origin of calli	Differentiation (%)	
		MS	N6
Tiyonishiki	Immature embryo	0	11
Miyazaki 25	Immature embryo	29	6
Toyonishiki	Immature embryo	*	0
Tou 108	Immature embryo	0	0
Miyazaki 29	Immature embryo	15	23
Kamenoo	Immature embryo	18	44
Shindaiokoshi	Immature embryo	0	0
G 88	Immature embryo	0	0
G 11	Immature embryo	*	0
Konansou	Immature embryo	44	46
Bluebelle	Immature embryo	0	0
IR 24	Immature embryo	14	*

* Culture lost before plants could be regenerated

$$\text{Differentiation ratio} = \frac{\text{No. of calli differentiated}}{\text{No. of calli inoculated}}$$

Table 2. Effects on differentiation of casein hydrolysate (CH) and proline in the liquid subculture medium. Calli were subcultured every 3 days in the liquid medium. Calli (about 1 mm in diameter) were transferred to the N6 solid medium that included 1 mg/l NAA and 5 mg/l 4-PU). Results were scored at the end of 14 days of culture on media used for differentiation

Medium	CH mg/l	Proline m*M*	No. of calli inoculated	Differentiation (%)
N6	0	0	30	53
N6	0	5	30	83
N6	0	10	30	77
N6	0	25	29	83
N6	300	0	29	41
N6	300	5	29	93
N6	300	10	29	89
N6	300	25	39	75
MS	*	*	*	*
MS	300	0	27	52
MS	300	25	36	51

* Cells died during subculture

$$\text{Differentiation ratio} = \frac{\text{No. of calli differentiated}}{\text{No. of calli inoculated}}$$

Table 3. Effect of intervals between subcultures. Calli were subcultured every 3 or 7 days in N6 medium that contained casein hydrolysate (300 mg/l), proline (10 m*M*), sucrose (30 g/l) and 2,4-D (1 mg/l). After 3 days, calli (about 1 mm in diameter) were transferred to N6 medium supplemented with NAA (1 mg/l), 4-PU (5 mg/l), sucrose (30 g/l) and Gelrite (2 g/l). Results were scored at the end of 14 days of culture on differentiation medium

Subculture interval (days)	No. of calli inoculated	No. of calli regenerated
3	10	10
	25	16
7	10	0

Table 4. Effect of size of cell clusters on differentiation. Calli were subcultured every 3 days in N6 medium supplemented with casein hydrolysate (300 mg/l), proline (10 m*M*), sucrose (30 g/l) and 2,4-D (1 mg/l). The clusters were sieved through nylon screen with a pore size of 25, 198, 423, and 680 μm. Cell clusters of each size were transferred to N6 medium that contained NAA (1 mg/l), kinetin (5 mg/l), sucrose (30 g/l) and Gelrite (2 g/l). After 14 days, the number of cell clusters that had differentiated was counted

Cluster size in μm	No. of calli inoculated	Differentiation (%)
25–198	280	0
198–423	285	19
423–680	160	23
680–	39	36

$$\text{Differentiation ratio} = \frac{\text{No. of cell clusters regenerated}}{\text{No. of cell inoculated}}$$

Table 5. Effect of the concentration of salts in the differentiation medium on differentiation. Calli were subcultured every 3 days in N6 medium supplemented with casein hydrolysate (300 mg/l), proline (10 m*M*), sucrose (30 g/l) and 2,4-D (1 mg/l). Cell clusters (198–423 μm in diameter) were washed and transferred to various dilution of N6 that contained NAA (1 mg/l), kinetin (5 mg/l) and sucrose (30 g/l) at a density of 25 clusters/ml

Medium	No. of cell clusters	Differentiation (%)
N6	130	0
1/2 N6	124	22
1/4 N6	107	22

$$\text{Differentiation ratio} = \frac{\text{No. of cell clusters regenerated}}{\text{Total no. of cell clusters}}$$

Table 6. Effects of 2,4-D and kinetin in the liquid differentiation media on differentiation. Calli were subcultured every three days in N6 liquid medium with 1 mg/l 2,4-D, 300 mg/l casein hydrolysate and 10 m*M* proline and then transferred to 1/2 N6 liquid media that contained sucrose (30 g/l) at a density of 25 clusters/ml. Casein hydrolysate and proline were not present in the media used for differentiation. After 14 days, the total number of cell clusters and the number of clusters that had regenerated were counted

2,4-D (mg/l)	Kinetin (mg/l)	No. of cell clusters	Differentiation (%)
0	0	246	9
0	0.01	113	6
0	0.1	132	16
0	1	67	0
0.001	0.01	697	13
0.001	0.1	467	15
0.001	1	263	6
0.01	0.01	369	14
0.01	0.1	504	10
0.01	1	396	8
0.1	0.01	567	4
0.1	0.1	472	3
0.1	1	238	0

$$\text{Differentiation ratio} = \frac{\text{No. of cell clusters regenerated}}{\text{Total no. of cell clusters}}$$

Size of clusters of cells and the frequency of differentiation

A correlation between differentiation and the size of the clusters of cells was also observed: larger clusters showed higher frequency of differentiation than smaller ones (Table 4).

Regeneration in liquid media

We tried to define a suitable medium for the regeneration of rice in suspension cultures. Clusters of cells did not

Table 7. Effects of NAA and kinetin in the liquid differentiation media on differentiation. Calli were subcultured every 3 days in N6 liquid medium with 1 mg/l 2,4-D, 300 mg/l casein hydrolysate and 10 m*M* proline and then transferred to 1/2 N6 liquid media that contained sucrose (30 g/l) at a density of 25 clusters/ml. Casein hydrolysate and proline were not present in the media used for differentiation. After 14 days, the total number of cell clusters and the number of clusters that regenerated were counted

NAA (mg/l)	Kinetin (mg/l)	No. of cell clusters	Differentiation (%)
0	0	246	9
0	0.01	113	6
0	0.1	132	16
0	1	69	0
0.01	0.01	378	16
0.01	0.1	262	13
0.01	1	343	11
0.1	0.01	440	11
0.1	0.1	466	7
0.1	1	371	13
1	0.01	549	4
1	0.1	422	5
1	1	374	11

$$\text{Differentiation ratio} = \frac{\text{No. of cell clusters regenerated}}{\text{Total no. of cell clusters}}$$

Table 8. Effects of NAA and 4-PU in the liquid differentiation media on differentiation. Calli were subcultured every 3 days in N6 liquid medium with 1 mg/l 2,4-D, 300 mg/l casein hydrolysate and 10 m*M* proline and then transferred to 1/2 N6 liquid media that contained sucrose (30 g/l) at a density of 25 clusters/ml. Casein hydrolysate and proline were not present in the media used for differentiation. After 14 days, the total number of clusters and the number of the clusters that regenerated were counted

NAA (mg/l)	4-PU (mg/l)	No. of cell clusters	Differentiation (%)
0	0	246	9
0	0.01	238	8
0	0.1	228	14
0	1	267	7
0.01	0.01	373	9
0.01	0.1	394	17
0.01	1	337	12
0.1	0.01	337	17
0.1	0.1	398	9
0.1	1	279	17
1	0.01	371	11
1	0.1	363	13
1	1	354	17

$$\text{Differentiation ratio} = \frac{\text{No. of cell clusters regenerated}}{\text{Total no. of cell clusters}}$$

regenerate in N6 medium that contained NAA (1 mg/l), kinetin (5 mg/l) and sucrose (30 g/l) but regenerated in half-strength N6 nutrient medium (½ N6) and in ¼ N6 media supplemented with NAA (1 mg/l), kinetin (5 mg/l) and sucrose (30 g/l) (Table 5).

Table 9. Effects of IAA and 4-PU in the liquid differentiation media on differentiation. Calli were subcultured every three days in N6 liquid medium with 1 mg/l 2,4-D, 300 mg/l casein hydrolysate and 10 m*M* proline and then transferred to 1/2 N6 liquid media that contained sucrose (30 g/l) at a density of 25 clusters/ml. Casein hydrolysate and proline were not present in the media used for differentiation. After 14 days, the number of cell clusters and the number of clusters that regenerated were counted

IAA (mg/l)	4-PU (mg/l)	No. of cell clusters	Differentiation (%)
0	0	238	8
0	0.1	228	14
0	1	267	7
0.01	0.01	208	5
0.01	0.1	246	5
0.01	1	223	12
0.1	0.01	242	4
0.1	0.1	249	6
0.1	1	246	8
1	0.01	239	7
1	0.1	222	7
1	1	237	14

$$\text{Differentiation ratio} = \frac{\text{No. of cell clusters regenerated}}{\text{Total no. of cell clusters}}$$

Table 10. Effect of the density of clusters on differentiation. Calli were subcultured every 3 days in N6 medium supplemented with casein hydrolysate (300 mg/l), proline (10 m*M*), sucrose (30 g/l) and 2,4-D (1 mg/l). Cell clusters (198–423 μ in diameter) were washed and transferred to 1/2 N6 medium supplemented with NAA (0.01 mg/l), 4-PU (0.1 mg/l) and sucrose (30 g/l)

Cluster density (clusters/ml)	No. of cell clusters	Differentiation (%)
6	85	36
13	89	37
25	136	18
50	417	16

$$\text{Differentiation ratio} = \frac{\text{No. of cell clusters regenerated}}{\text{Total number of cell clusters}}$$

Hormonal conditions suitable for regeneration were also investigated in liquid media (Tables 6–9). High concentrations of auxins were inhibitory for regeneration, but the calli underwent necrosis in auxin-free medium. Cytokinins were effective for regeneration, but necrosis occured at high concentrations of cytokinins. The effects of the density of clusters on regeneration are shown in Table 10, and the density of clusters was found to be one of the most important factors in regeneration. At densities of more than 25 clusters/ml the frequency of regeneration decreased markedly. At densities between 6 clusters/ml and 13 clusters/ml we observed the highest frequency of regeneration. We investigated the effects of sucrose on differentiation at various concentrations

Table 11. Effect of sucrose on differentiation. Calli were subcultured every 3 days in liquid N6 medium that contained casein hydrolysate (300 mg/l), proline (10 m*M*), sucrose (30 g/l) and 2,4-D (1 mg/l). Then cell clusters (198–423 μm in diameter) obtained by sieving calli through nylon screen with between 198-μm and 423-μm pores were washed and transferred to 1/2 N6 medium supplemented with NAA (0.01 mg/l) and 4-PU (0.1 mg/l) at a density of 13 clusters/ml. After 14 days, total number of cell clusters and the number of differentiated clusters were counted

Sucrose concentration (g/l)	No. of calli	Differentiation (%)
2.25	268	33
3	295	36
4.5	315	48
6	392	40
7.5	352	25
9	342	24

$$\text{Differentiation ratio} = \frac{\text{No. of cell clusters regenerated}}{\text{Total no. of cell clusters}}$$

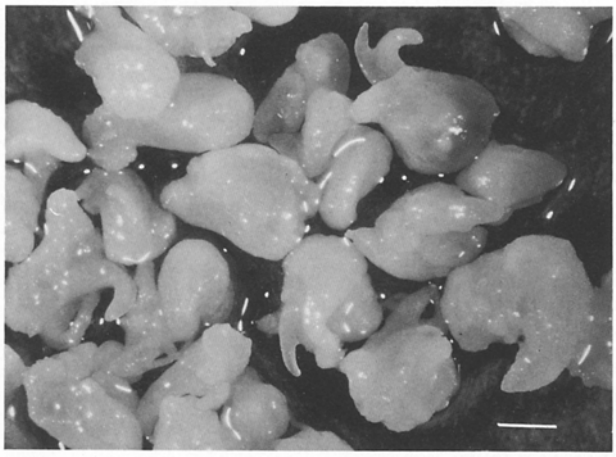

Fig. 1. Rice somatic embryos induced in 1/2 N6 liquid medium supplemented with NAA (0.01 mg/l), 4-PU (0.1 mg/l) and sucrose (45 g/l) at a density of 13 clusters/ml. Bar indicates 500 μm

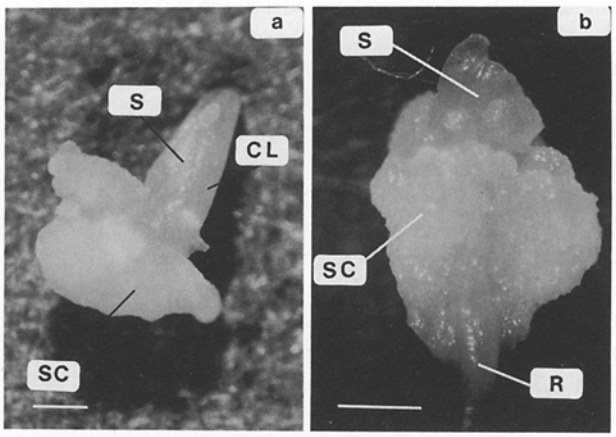

Fig. 2a and b. A typical somatic embryo induced in the same medium as described in the legend to Fig. 1. SC: scutellum; S: shoot; CL: coleoptile; R: radicle. Bar indicates 500 μm

(Table 11). The highest frequency of regeneration was observed when 4.5% sucrose was used. The frequency of regeneration reached about 50% in ½ N6 medium that contained NAA (0.01 mg/l), 4-PU (0.1 mg/l) and sucrose (30 g/l) at a density of 13 clusters/ml.

Morphology

Figure 1 shows differentiating clusters of cells in liquid ½ N6 medium supplemented with NAA (0.01 mg/l), 4-pyridylurea (4-PU) (0.1 mg/l) and sucrose (45 g/l). A distinct coleoptile, scutellum and radicle were observed in most cases, as shown in Fig. 2, indicating a dipolar structure characteristic of embryos. We confirmed the dipolar structure of differentiating clusters by anatomical examination of cross sections (data not shown). In a differentiating cluster shown in Fig. 2a, a radicle is not visible, but it could be clearly observed in sections of the cluster. Our data indicate that regeneration occurred via embryogenesis. Detailed morphology of the process of regeneration will be reported elsewhere.

Discussion

In an attempt to establish a high-frequency embryogenesis system for rice, we first selected cell lines which have a high potential for regeneration. In rice, as well as in other species, genotype has been considered to be an important factor affecting the potential for regeneration in vitro (Duncan 1985). Our results support this view (Table 1). Some cultivars showed a superior ability to regenerate while others did not. Since the calli initiated from immature embryos of Konansou had the greatest ability to regenerate, we used this cultivar in subsequent experiments.

Suspension cultures are more suitable for physiological and biochemical investigations of differentiation than callus cultures grown on solid media, because the former provide a more homogeneous system than the latter. However, it has been reported that it is difficult to maintain the ability to regenerate in liquid suspension cultures of Gramineae (Vasil and Vasil 1985b). It has been suggested that the subculture medium is an important factor in the maintenance of the potential for regeneration, and MS medium has been reported to be suitable for regeneration of Gramineae (Vasil and Vasil 1985b).

In our experiments, modified MS medium (R-MS medium) did not support the growth of cells in suspension cultures (Table 2). However, if R-MS medium was supplemented with casein hydrolysate and/or proline, cells were able to grow and their ability to regenerate was maintained for more than 18 months. The frequency of regeneration reached about 50% when cells were transferred to

N6 regeneration medium that contained NAA (1 mg/l), kinetin (5 mg/l), sucrose (30 g/l) and Gelrite (2 g/l). It was found that N6 medium alone was more suitable for growth of cells during subculturing; cells grew vigorously in the absence of casein hydrolysate and proline and the frequency of regeneration was rather high (about 50%). The frequency of regeneration and the efficiency of cell growth were increased markedly if the N6 medium was supplemented with proline (Table 2), while addition of casein hydrolysate only slightly increased the frequency of regeneration. Proline was reported to increase the formation of friable embryogenic calli of *Zea mays* and other species (Armstrong and Green 1985; Sozinov et al. 1981). The role of proline in growth and differentiation is not clear (Vasil and Vasil 1986; Trigiano and Conger 1987), and further research is necessary to elucidate the mechanism of stimulation by proline.

The interval between subculturing was the most important variable obtaining a high frequency of regeneration (Table 3). Using suspension cultures of Graminae, Chandler and Vasil (1984) found that the subculture interval is an important factor in the preparation of embryogenic suspension cultures of *Pennisetum purpureum*; they further found that reducing the subculture interval caused a doubling of the number of pieces of callus that eventually produced embryogenic callus, probably via a reduction in the conversion rate of embryogenic cells to non-embryogenic cells. However, in our experiments, subculturing at intervals of 3 days induced regeneration at high frequency, while subculturing at intervals of 7 days did not result in any embryogenesis at all. These results suggest the possibility that some substance (s) which inhibited regeneration was secreted into the medium, and that subculturing after a short interval has the effect of diluting the inhibitor(s). The fact that the addition of charcoal to media promotes regeneration supports this view (Peck and Cumming 1986).

Thus, in subsequent experiments, cells were subcultured in N6 medium supplemented with casein hydrolysate (300 mg/l), proline (10 m*M*), sucrose (30 g/l) and 2,4-D (1 mg/l) at intervals of 3 days.

As previously mentioned, a correlation between differentiation and the size of the cluster of cells was observed: the larger the clusters, the higher the frequency of differentiation (Table 4). Although the reason for this correlation is not known, we used the smallest size of the clusters (198–423 μm) which were able to differentiate in all subsequent experiments, because smaller clusters are more suitable for biochemical investigations of differentiation than larger ones.

To our knowledge, there have been no reports of embryogenesis of rice in liquid medium. Since suspension cultures are more suitable for investigations of differentiation, we attempted to induce differentiation of clusters of rice cells in suspension cultures. The calli did not differentiate in liquid N6 medium, but did differentiate in ½ N6 and ¼ N6 medium (Table 5), indicating that the concentration of salts in N6 medium is too high to allow differentiation.

Many authors have reported that various combinations and concentrations of hormones have marked effects on regeneration. In our rice cultures, however, the frequency of regeneration did not change markedly with various combinations of hormones (Tables 6–9), but the concentrations of hormones had notable effects on differentiation. High concentrations of auxin and cytokinin did not favor differentiation. Cells could grow but did not differentiate in media supplemented with auxins at high concentrations. Cytokinin at high concentrations did not support growth, but caused necrosis. A marked difference in effects on differentiation was not observed between individual hormones. The highest frequency of differentiation was observed in ½ N6 medium supplemented with NAA (0.01 mg/l), 4-PU (0.1 mg/l) and sucrose (3%) (Table 8). It has been reported that transfer to hormone-free medium results in differentiation in Gramineae (Vasil and Vasil 1985a, b). However, in our suspension cultures of rice cells, a higher frequency of differentiation was observed after transfer of cells to medium that contained auxin and cytokinin. The reason for the discrepancy in the response to hormones is unknown.

The density of clusters in the medium was also an important factor in regeneration (Table 10). A lower density of clusters was more suitable for the induction of differentiation than a higher density. If an inhibitor(s) of regeneration is secreted into the medium, as appears possible from the promotion of regeneration by subculture at frequent intervals, more of such an inhibitor(s) may be secreted into the media at a higher density of clusters than at a lower one.

It has been reported that a high concentration of sucrose, for example, 9% (w/v) has marked promotional effects on regeneration (Green 1982), but such a noted effect was not observed in our experiments (Table 11). The highest frequency of regeneration was observed in the ½ N6 medium that contained 4.5% sucrose.

There have been few detailed reports on the morphology of regeneration of rice calli. Some researchers have reported that regeneration occurs via embryogenesis in rice cultures (Chen et al. 1985; Abe and Futsuhara 1985), but the embryos differentiated from large calli and radicles were not observed. Thus, we cannot conclude from these reports of others that regeneration in rice cultures occurs via embryogenesis, in a manner different from regeneration of other Graminae such as *Zea mays* (Vasil et al. 1985), *Panicum maximum* (Lu and Vasil 1985) and *Triticum aestivum* (Ozias-Akins and Vasil 1982). Since regeneration in liquid medium occurred from a whole clusters of cells but not from parts of the cell clusters in liquid medium in the experimental system re-

ported here, as shown in Fig. 1, it is easy to follow the pathway of regeneration. We were able to see clearly in regenerated clusters of cells many of the characteristic organs of rice, such as the scutellum, coleoptile, coleorhyiza, radicle and the vascular bundle leading to the scutellum (data not shown). Therefore, differentiation in this system can be regarded as embryogenesis rather than organogenesis. The details of the morphology will be reported elsewhere.

In summary, we have been able to establish a high-frequency embryogenesis system in suspension cultures of rice. The frequency reached 90% on solid medium and 50% in liquid medium. This is the first report of the induction of embryogenesis in suspension cultures of rice.

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