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Efficient Fertile Plant Regeneration from Protoplasts of Javanica Rice and Their Ploidy Determination by Flow Cytometry

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Javanica 벼 원형질체로 부터 효율적인 식물체 재분화와 flow cytometry에 의한 ploidy 검정

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The Southeast Asian javanica rice variety Tinawen was investigated for efficient protoplast culture and plant regeneration from cell suspension-derived protoplasts using a feeder cell culture method. Feeder cells of both *Lolium multiflorum* and *Oryza ridleyi*, either alone, or in combination, were employed and plants were regenerated from protoplast-derived colonies on several plant regeneration media. Dehydration of protoplast-derived colonies was also investigated as a means of enhancing plant regeneration. In the presence of *L. multiflorum* or *O. ridleyi* feeder cells, the protoplast plating efficiency ranged from 0.09% to 1.48%, depending on the feeder cell type and the age of the cell suspension. *L. multiflorum* feeder cells induced approximately 6-fold higher plating efficiency compared with those of *O. ridleyi*. The plant regeneration frequencies were 19.3-31.7% with *L. multiflorum*, 13.0-18.0% with *O. ridleyi* and 18.0-22.0% with a mixture of both in various plant regeneration media when protoplast-derived colonies were dehydrated, while for the non-dehydrated colonies, the values were 2.0-7.0%, 3.0-5.0% and 0-4.0%, respectively. Flow cytometric analysis of 34 protoplast-derived plants showed that the majority of plants were diploids and only 2 plants were tetraploids. The plants which were transferred to glasshouse were fertile.

Key words: feeder cells, flow cytometric analysis, *Oryza sativa* L., protoplast culture.

Protoplast technology plays a very important role in both somatic hybridization and genetic transformation for crop improvement. However, in order that protoplast techniques can be of real value as an aid to rice breeding, efficient and reproducible protocols for regeneration of fertile plants from protoplasts are necessary for all rice varieties (Cocking, 1993). Among the three sub-species of *Oryza sativa*: indica, japonica and javanica, most researches so far have been concentrated on indica and japonica varieties, which have also been benefited most from the improvement of plant biotechnological techniques during the past few decades. In these two sub-species, several groups have successfully regenerated plants from protoplasts (Abdullah et al., 1986; Kyozuka et al., 1987; Lee et al., 1989; Torrizo and Zapata, 1992; Jain et al., 1995; Lee et al., 1997a). However, there have been few reports on protoplast culture and plant regeneration of javanica rices. Coulibaly and Demarly (1986) reported the first successful regeneration of plantlets from protoplasts isolated from callus derived from the base of the coleoptile of the javanica rice variety Moroberekan. This variety originated in Cote d' Ivoire of Africa (Guiderdoni et al., 1992). Wang et al. (1989) isolated protoplasts from suspensions of the two Southern U.S.

long-grain javanica varieties Gulfmont and Lemont, and cultured the protoplasts using the agarose embedding procedure without feeder cells. Green shoots only were obtained from Gulfmont. Plant regeneration from protoplasts was also successfully achieved in the Southern U.S. long-grain javanica rice varieties Texmont and Labelle (Suh et al., 1992; Li et al., 1992). However, there have been no reports, as yet, of plant regeneration from protoplasts of Southeast Asian javanica varieties. Rice is the most important staple food in the developing world. Breakthroughs in rice breeding have already been accomplished for irrigated lowland and rainfed paddy varieties, and to some extent for upland varieties. The higher elevation areas in the rice terraces of the Cordillera are of special interest to rice breeders in the Philippines. Traditional low yielding cultivars remain principal varieties despite 20 years of breeding effort by scientists at the International Rice Research Institute. The javanica rice variety Tinawen is a traditional cultivar grown by farmers in this area. Establishment of tissue and protoplast culture techniques of this cultivar are essential and pre-requisite to more advanced genetic manipulations.

In this paper, we report an efficient procedure for protoplast culture system using the filter membrane procedure with feeder cells and plant regeneration from protoplast-derived callus in the Southeast Asian javanica rice variety Tinawen and also ploidy level of protoplast-derived plants using flow cytometry.

MATERIALS AND METHODS

Plant materials

Mature seeds of the Southeast Asian javanica rice (*Oryza sativa* L.) variety Tinawen were obtained from the Philippines Rice Research Institute. *Lolium multiflorum* cell suspensions for feeder cells were supplied by Dr. E. Guiderdoni (IRAT-CIRAD, Montpellier, France) and were maintained in N6 medium (Chu et al., 1975) supplemented with 2.0 mg/L 2,4-D. *Oryza ridleyi* cell suspensions were initiated using callus developed from mature-seed scutellum and maintained in MS medium (Murashige and Skoog, 1962) containing 2.0 mg/L 2,4-D as described previously (Jain et al., 1995). Both feeder cell suspensions were incubated on a rotary shaker (120 rpm) at 27°C in the dark with weekly subculture.

Callus induction

Dehusked mature seeds were sterilized by soaking in 30% (v/v) solution of 'Domestos' commercial bleach for 30 min. and rinsed five times with sterile water. The seeds were placed onto the surface of LS medium (Linsmaier and Skoog, 1965) semi-solidified with 0.4% (w/v) Sea Kem agarose (FMC Bio Products, Rockland, ME, USA), supplemented with 3% (w/v) sucrose and 2.5 mg/L 2,4-D (LS 2.5), and incubated at 27°C in the dark.

Initiation and maintenance of cell suspension cultures

Cell suspension cultures were initiated by transferring 1.0 g fresh weight of friable, globular embryogenic callus from 4 week-old primary callus to 20 ml of filter sterilized amino acid based liquid medium containing 2.0 mg/L 2,4-D, 0.1 mg/L GA_3 and 0.2 mg/L kinetin (AA2; Abdullah et al., 1986) in 100 ml Erlenmeyer flasks. The cultures were maintaind on a rotary shaker at 120 rpm in the dark at 27°C. Cell suspension cultures were subcultured using the same volume of fresh AA2 medium daily during the first week, followed by 3-4 d intervals for 12 months, removing all the conditioned medium and replacing it with the fresh medium. After 12 months, 1 ml packed cell volume (pcv) of cell suspension was subcultured using 24 ml of fresh AA2 medium and 3 ml of conditioned medium from existing cultures in 100 ml Erlenmeyer flask at weekly intervals. Cell suspension cultures were suitable for protoplast isolation in 12 months after initiation.

Protoplast isolation and culture

Protoplasts were isolated and their viability and purity were determined essentially as described by Abdullah et al. (1986). Non-embryogenic cell suspensions of *L. multiflorum* and *O. ridleyi* were used as feeder cells for nurse cultures either alone or in combination as described by Jain et al. (1995). For the nurse plates, 0.5 ml of pcv from a suspension of either *L. multiflorum* or *O. ridleyi*, or a 1:1 (v:v) mixture of both, was suspended in 10 ml of KPR medium containing 0.8% (w/v) Sea Plaque agarose (FMC Bio Products) into a 9 cm Petri dish. The nurse plates were prepared 1 d prior to protoplast isolation and cultured in the dark at 27°C. Two cellulose nitrate membrane filters (47 mm diameter, 0.2 μm pore size, Whatman International Ltd., Maidstone, UK) were used in each petri dish. An intact membrane and two half

membranes were laid side by side on the surface of the medium with or without embedded-feeder cells. Aliquots of 100 μl and 200 μl containing protoplasts (0.5×10^5 ml^{-1}) were placed onto the surface of each half and intact membrane, respectively, and spread uniformly using a sterilized plastic bacterial inoculation loop. The plates were incubated in the dark at 27°C. After 21-28 d of culture, membranes were transferred to the surface of 0.4% (w/v) agarose-solidified LS2.5 medium and incubated for 14 d in the dark at 27°C. The plating efficiency of these cultures was determined after 35 d of culture based on the percentage of protoplast that had undergone sustained division leading to visible colony formation.

Plant regeneration

Protoplast-derived colonies, 1-2 mm in diameter, were transferred to 25 well plastic square dishes with each well containing 2 ml of MS-based plant regeneration medium supplemented with different auxin-cytokinin combinations with either 3.0% (w/v) sucrose or 3.0% (w/v) maltose, or a combination of 1.5% (w/v) sucrose and 1.5% (w/v) maltose, solidified with 4.0 or 10.0 g/L Sea Kem agarose (Table 2). Two colonies were transferred to each well. Cultures were incubated in the dark at 27°C for 14 d and then transferred to the light (55 mol m^{-2} sec^{-1}, daylight fluorescent tubes), under a 16 h photoperiod at 27°C. Callus cultured on media supplemented with 10.0 g/L agarose were transferred to the corresponding medium containing 4.0 g/L agarose before transferring them to the light. Plant regeneration frequency was recorded 28 d after transfer to regeneration medium and was calculated as the percentage of protoplast-derived colonies which formed plants. Plants regenerated from protoplasts were transferred to MS based micropropagation medium supplemented with 2 mg/L BAP, 50.0 g/L sucrose and 4.0 g/L Sea Kem agarose (MSBP) and incubated at 27°C in the light for 28 d. Shoots were then individually transferred to MS based medium containing 1.5 mg/L NAA, 30.0 g/L sucrose and 4.0 g/L Sea Kem agarose (MSN1.5) and incubated at 27°C in the light to induce root formation. Young regenerated plants were transplanted to pots and maintained in the glasshouse.

Determination of ploidy level of the regenerants by flow cytometry

The javanica rice variety Tinawen seedlings from mature seeds (2-week old) were grown in jars with MS medium solidified with 0.8 % (w/v) agar and used as a source of diploid nuclei. Leaf samples from 34 protoplast-derived regenerated plants were randomly selected from plants grown in MSN1.5 medium. Approximately 1 g of fresh leaf material was removed from each sample and chopped gently into 1-2 mm strips with a scalpel in staining buffer (15 mM Tris HCl, 2 mM EDTA, 0.5 mM spermine, 80 mM KCl, 20 mM NaCl, 15 mM DTT, 0.1% Triton X-100, pH 7.5) (Dolezel et al., 1989). The isolated nuclei were separated from tissue debris by passing through a 30 μm nylon sieve, mixed with a small aliquot of fluorescently labelled beads (Immuno-Checked Beads, Coulter Electronics Ltd., Luton, U.K.), then introduced into the flow cytometer. Fifty ml of a solution of fluorescently labelled 'Immuno-Check' polystyrene beads (Coulter Electronics Ltd, Luton, UK) were added as an internal fluorescence standard. Fluorescence measurement was performed using a Coulter EPICS 541 flow cytometer. The argon-ion laser was turned on to produce 100 mW output at a wavelength of 488 nm. Ten thousand particles were analyzed per sample: single parameter green fluorescence histograms (log scale) were acquired and stored on disk. The histograms were transferred to an IBM PC computer and the relative mean linear fluorescence values of the G0/G1 nuclei were determined using programmes written by N.W. Blackhall. Adjustments to these values were made to compensate for variations in the values obtained for the fluorescent beads.

RESULTS

Callus production and establishment of cell suspensions

Mature seeds readily formed two types of callus at the scutellar surface when germinated on LS2.5 medium. A non-embryogenic, root-producing callus which was wet in appearance and often mucilaginous was unsuitable for the initiation of cell suspensions. The other type, embryogenic callus, was dry, compact, friable and globular in appearance and was found to be most suitable for the initiation of cell suspensions. The cell suspensions initiated using friable embryogenic callus in AA2 medium were highly glutinous and had to be frequently subcultured to maintain their growth. Washing of cell suspensions with fresh medium at every subculture was necessary to remove such glutinous material. It took one year to establish fine, non-sticky suspensions,

consisting of cells with a dense cytoplasm. At this stage, the cells in the suspension were actively dividing and the proportion of these dividing groups of cells gradually increased with a doubling time of 3-4 d. A weekly subculture cycle was found suitable for the maintenance of these suspension cultures. For subculture, the ratio of inoculum, fresh medium and conditioned medium exerted a major effect on the growth of suspension cultures. It was observed that a minimum of 1ml pcv of small cell colonies, 3 ml of conditioned medium from the existing culture and 24 ml of fresh medium was essential to maintain the suspension cultures.

Protoplast isolation and culture

The yield of protoplasts was 6.7-8.2 × 10^6 g f. $wt.^{-1}$ from 13-20 month-old cell suspensions. Freshly isolated protoplasts were densely cytoplasmic and their size ranged from 8-25 μm diameter (Figure 1a). Staining with FDA showed that on the average about 90% of the protoplasts was viable (Figure 1b). Glutinous cell suspensions less than 12 month-old failed to release protoplasts. The protoplasts were cultured in KPR medium using filter membrane procedure. In order to examine the division of protoplasts after 5 days of culture, the membranes with protoplasts were taken out and washed in CPW 13M solution. The protoplasts in CPW 13M solution were examined under inverted microscope. Most of the protoplasts were found to undergo first and second divisions. Third division was observed within 1 week of culture. Microcolonies developed after 3 weeks of culture. At this stage, transfer of membranes to LS2.5 medium was necessary for further development of protoplast-derived cells into micro-callus. Micro-callus of 1-2 mm in diameter were obtained in LS2.5 medium after 5 weeks of culture (Figure 1c). At this stage, it was essential to transfer micro-callus to shoot regeneration medium (Figure 1d) When protoplasts were cultured on the surface of the membrane filters using Sea Plaque agarose embedded feeder cells of either *L. multiflorum* or *O. ridleyi*, or a 1:1 mixture of these cells, there was a sustained division leading to colony formation. In the presence of *L. multiflorum* or *O. ridleyi* feeder cells, the protoplast plating efficiency ranged from 0.09 to 1.48%, depending on the feeder cells and age of cell suspensions used for protoplast isolation. In the case of protoplasts isolated from 20-month-old cell suspensions, *L. multiflorum* feeder cells gave about 6-fold higher plating efficiency compared with cells of *O. ridleyi* cell. In combination, *O. ridleyi* and *L. multiflorum* feeder cells resulted in a protoplast plating efficiency of 0.8% compared to that of 1.5% by *L. multiflorum* alone as feeder cells. However, when protoplasts were cultured on a membrane without feeder cells, formation of visible cell colonies was not observed (Table 1).

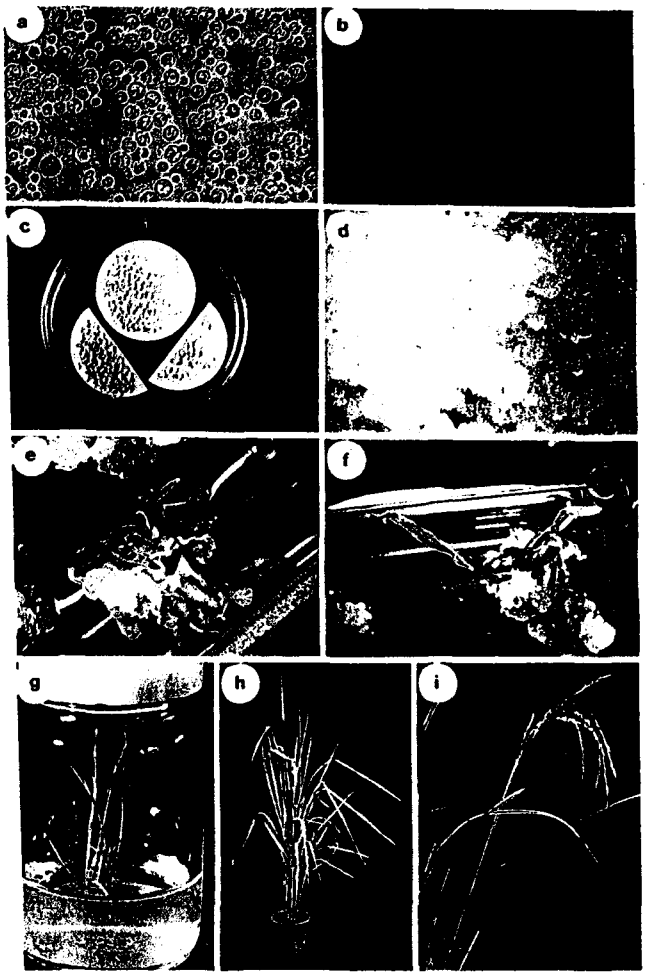

Figure 1. Plant regeneration from cell suspension-derived protoplasts of the javanica rice variety Tinawen. a) Freshly isolated cell suspension-derived protoplasts. b) Viable protoplasts stained with FDA. c) Protoplast-derived colonies cultured on membranes for 2 weeks after the transfer of membranes to LS2.5 medium without feeder cells. d) Protoplast-derived colonies growing on the surface of a filter membrane after 5 weeks of culture. e) Differentiation of plantlets from protoplast-derived callus after 4 weeks of culture on MSKN medium with 30 g/L maltose. f) Differentiation of plantlets from protoplast-derived callus after 4 weeks of culture on MSKN medium containing 15 g/L sucrose and 15 g/L maltose. g) Development of plantlets from protoplast-derived callus after transfer to MSBP medium. h) A morphologically normal plant regenerated from protoplasts and transferred to the glasshouse. i) A fertile plant regenerated from protoplasts and transferred to the glasshouse.

Plant regeneration from protoplast-derived colonies

Table 1. Plating efficiency from cell suspension-derived protoplasts of the javanica rice variety Tinawen using the filter membrane culture procedure

Age of cell suspension (months)	Protoplast yield ($\times 10^6$/gf.wt.)	Feeder cells (0.5 pcv/10 ml medium)	Plating efficiency (%)
13	7.5	None	0.00 ± 0.00
		L. multiflorum	0.09 ± 0.02
15	8.2	None	0.00 ± 0.00
		L. multiflorum	0.59 ± 0.18
20	6.68	None	0.00 ± 0.00
		L. multiflorum	1.48 ± 0.05
		O. ridleyi	0.25 ± 0.10
		L. multiflorum + *O. ridleyi*	0.83 ± 0.15

Mean ± S.E. of 3 replicates
Plating efficiency was expressed as the percentage of protoplasts forming visible colonies after a culture period of 35 d.

Table 2. Composition of various MS-based plant regeneration media used for shoot regeneration from cell suspension-derived protoplasts of the javanica rice variety Tinawen

Composition	RM1	RM2	RM3	RM4	RM5	RM6	RM7	RM8	RM9
Growth regulators (mg/L)									
NAA	0.5	0.5	0.5	0.5	0.5	0.5	1	1	0.5
Kinetin	2	2	2	2	2	2	8	8	0
BAP	0	0	0	0	0	0	0	0	2
Carbohydrate sources (g/l)									
Sucrose	30	30	0	0	15	15	30	0	30
Maltose	0	0	30	30	15	15	0	30	0
Agarose	4	10	4	10	4	10	4	4	4

RM: regeneration medium

Plant regeneration from protoplast-derived colonies was investigated using various MS-based plant regeneration media with sucrose, maltose or a mixture of both as carbohydrate source (Table 2). Protoplast-derived colonies underwent somatic embryogenesis and produced plants after 2-4 weeks of culture on regeneration media (Figure 1e, f). The plant regeneration frequency depended on the auxin/cytokinin composition, carbohydrate source and agarose concentration (Table 3). Maltose-containing media were superior for regeneration of green plants from protoplast-derived colonies of the javanica rice Tinawen. The media containing both kinetin (2.0 mg/L) and NAA (0.5 mg/L) supplemented with either 3% maltose or a combination of 1.5% maltose and 1.5% sucrose and semi-solidified with 1% agarose, proved to be

Table 3. Effect of different media and feeder cells on plant regeneration from protoplast-derived colonies of the javanica rice variety Tinawen.

Feeder cells used for protoplast culture	Regeneration medium	No. of callus transferred	No. of callus regenerating plants	Plant regeneration frequency (%)
L. multiflorum	RM1	500	11	2.2
L. multiflorum	RM2→RM1*	300	58	19.3
L. multiflorum	RM3	600	44	7.3
L. multiflorum	RM4→RM3*	400	119	29.7
L. multiflorum	RM5	600	12	2.0
L. multiflorum	RM6→RM5*	400	127	31.7
L. multiflorum	RM7	300	3	1.0
L. multiflorum	RM8	300	26	8.7
L. multiflorum	RM9	300	0	0
O.ridleyi	RM3	100	5	5.0
O.ridleyi	RM4→RM3*	100	18	18.0
O.ridleyi	RM5	100	3	3.0
O.ridleyi	RM6→RM5*	100	13	13.0
L. multiflorum + *O.ridleyi*	RM3	100	4	4.0
L. multiflorum + *O.ridleyi*	RM4→RM3*	100	22	22.0
L. multiflorum + *O.ridleyi*	RM5	100	0	0
L. multiflorum + *O.ridleyi*	RM6→RM5*	100	18	18.0

* Callus were dehydrated in RM2, RM4 and RM6 media, respectively, for 2 weeks, then transferred to RM1, RM3 and RM5 media.

optimum dehydration medium for plant regeneration. The regeneration frequency increased significantly when protoplast-derived colonies were cultured on a regeneration medium with 1% agarose instead of the normal concentration of 0.4% agarose during the first two weeks of culture in the dark followed by culture with 0.4% agarose medium in the light. Plant regeneration frequency ranged from 19.3% to 31.7% with *L. multiflorum*, 13.0% to 18.0% with *O. ridleyi* and 18.0% to 22.0% when a mixture of both feeder cells were employed for protoplast-derived colonies which were subsequently stressed by exposure to 1% agarose. Without the dehydration treatment, the frequences were 2.0% to 7.3%, 3.0% to 5.0% and 0% to 4.0%, respectively (Table 3). Thus, these results suggest that dehydration treatment also increases plant regeneration from protoplast-derived colonies in javanica rice. Plants regenerated from protoplasts were cultured in MSBP medium containing 2 mg/L BAP and 50.0 g/L sucrose (Figure 1g), and then transferred to MS medium supplemented with 1.5 mg/L NAA and 30.0 g/L sucrose at 27°C in the light for root differentiation (MSN1.5). These plants flowered within 4 months of transfer to the glasshouse and were fertile (Figure 1h, i).

Ploidy determination of protoplast-derived plants by flow cytometry

Table 4. Ploidy level, as determined by flow cytometry, of javanica rice plants regenerated from protoplast-derived colonies.

Sample No.	Feeder cells used for protoplast culture	Relative fluorescence 1st exp.	2nd exp.	3rd exp.	Mean of relative* DNA content	Ploidy
Control	None	356	322	304	1.0 ± 0.0	2n
A1	*L. multiflorum*	374	283	371	1.0 ± 0.1	2n
A2	*L. multiflorum*	358	155	366	0.9 ± 0.2	2n
A3	*L. multiflorum*	351	254	342	1.0 ± 0.1	2n
A4	*L. multiflorum*	359	234	359	1.0 ± 0.1	2n
A5	*L. multiflorum*	373	260	344	1.0 ± 0.1	2n
A6	*L. multiflorum*	677	342	679	1.7 ± 0.3	4n
A7	*L. multiflorum*	377	298	348	1.0 ± 0.1	2n
A8	*L. multiflorum*	372	264	358	1.0 ± 0.1	2n
A9	*L. multiflorum*	391	273	361	1.0 ± 0.1	2n
A10	*L. multiflorum*	377	315	382	1.1 ± 0.1	2n
A11	*L. multiflorum*	371	245	368	1.0 ± 0.1	2n
A12	*L. multiflorum*	367	283	396	1.1 ± 0.1	2n
A13	*L. multiflorum*	367	277	343	1.0 ± 0.1	2n
A14	*L. multiflorum*	742	713	735	2.2 ± 0.1	4n
A15	*L. multiflorum*	374	359	358	1.1 ± 0.1	2n
B1	*L. multiflorum* + *O. ridleyi*	382	339	366	1.1 ± 0.1	2n
B2	*L. multiflorum* + *O. ridleyi*	360	234	370	1.0 ± 0.1	2n
B3	*L. multiflorum* + *O. ridleyi*	365	258	349	1.0 ± 0.1	2n
B4	*L. multiflorum* + *O. ridleyi*	338	290	351	1.0 ± 0.1	2n
B5	*L. multiflorum* + *O. ridleyi*	362	329	382	1.1 ± 0.1	2n
B6	*L. multiflorum* + *O. ridleyi*	354	334	386	1.1 ± 0.1	2n
B7	*L. multiflorum* + *O. ridleyi*	371	304	376	1.0 ± 0.1	2n
B8	*L. multiflorum* + *O. ridleyi*	378	309	373	1.1 ± 0.1	2n
B9	*L. multiflorum* + *O. ridleyi*	377	323	400	1.1 ± 0.1	2n
B10	*L. multiflorum* + *O. ridleyi*	380	335	387	1.1 ± 0.1	2n
B11	*L. multiflorum* + *O. ridleyi*	367	258	382	1.0 ± 0.1	2n
B12	*L. multiflorum* + *O. ridleyi*	372	354	375	1.1 ± 0.1	2n
B13	*L. multiflorum* + *O. ridleyi*	394	277	383	1.1 ± 0.1	2n
B14	*L. multiflorum* + *O. ridleyi*	377	253	387	1.0 ± 0.1	2n
B15	*L. multiflorum* + *O. ridleyi*	387	294	387	1.1 ± 0.1	2n
C1	*O. ridleyi*	394	260	407	1.1 ± 0.1	2n
C2	*O. ridleyi*	398	304	413	1.1 ± 0.1	2n
C3	*O. ridleyi*	390	251	413	1.1 ± 0.1	2n
C4	*O. ridleyi*	377	241	175	0.8 ± 0.1	2n

Rice (*Oryza sativa*) seedlings contain 1.2 pg DNA per 2C nucleus (Bennett and Smith, 1976)
*Mean ± S.E. of 3 replicates.

Determination of plant ploidy level using flow cytometry was performed by comparing the DNA content of plant samples from seedlings grown from mature seeds as the standard diploid plant. The nuclear DNA content of rice plants (*Oryza sativa* L.) has been standardized as 1.2 pg DNA per 2C nucleus (Bennett and Smith, 1976). Flow cytometric analysis of the 34 randomly selected protoplast-derived plants showed that the majority of plants were diploidy (32 plants) and only 2 plants were tetraploidy (Table 4).

DISCUSSION

Javanica rice is identified as a sub-species of *Oryza sativa* L. on the basis of morphology and geographical distribution. It is grown as typical upland rice from Africa and America and most upland rices from Southeast Asia. Glaszmann (1987) investigated the genetic structure of *Oryza sativa* L. using enzyme variation for classification of Asian rice varieties and identified 6 enzymatic groups (group I to group VI). Most javanica rice varieties belong to group VI, which is found mostly in temperate areas and high elevation areas in the tropics (most upland rice from Southeast Asia) (Glaszmann, 1987). The javanica rice variety Tinawen used in this experiment may belong to group VI because this

variety was a popular old traditional cultivar grown by farmers in terraces of high elevation areas in Southeast Asia.

Embryogenic callus can be used to initiate fine cell suspension cultures which have a high potential for plant regeneration. Establishment of such cultures is the critical step in successful plant regeneration from protoplasts in rice (Abdullah et al., 1986: Toriyama et al., 1986: Lee et al., 1989). It is likely that culture medium is one of the main factors controlling establishment of fine embryogenic suspension cultures. However, it is still difficult to find suitable medium for inducing fine embryogenic cell suspension cultures particularly in some varieties of indica rice (Lee et al., 1989). AA medium has been used to initiate and maintain cell suspension for protoplast isolation and culture in rice (Abdullah et al., 1986) In this study, the cell suspensions initiated using embryogenic callus in AA2 medium were highly glutinous and required frequent subculture to maintain cell growth. It took one year to establish fine non-sticky cell suspensions. Other studies from this laboratory and other laboratories have shown that a long period in culture is often associated with a loss of regeneration potential. Thus, there is still a need to find suitable culture medium for establishing fine non-sticky cell suspension within a short time. Several culture methods have been developed for rice protoplasts such as agarose embedding culture (Abdullah et al., 1986), nurse culture (Lee et al., 1989: Jain et al., 1995), mixed nurse culture (Kyozuka et al., 1987: Ghosh Biswas and Zapata, 1993) and liquid medium culture (Toriyama et al., 1986). In this experiment, protoplasts were cultured on the membrane filter with or without embedded-feeder cells and protoplast-derived microcolonies were obtained only from culture with feeder cells. Feeder cells have been reported to be beneficial or even essential for colony formation from rice protoplasts (Kyozuka et al., 1987: Lee et al., 1989: Ghosh Biswas and Zapata, 1993: Jain et al., 1995: Lee et al., 1997b). The feeder cells are generally used to maintain a critical density of active cells, which produce sufficient levels of putative growth promoting factors necessary for sustained protoplast division (Eigel and Koop, 1989). In the present study, *L. multiflorum* and *O. ridleyi* cells were essential for the formation of visible colonies from protoplasts of javanica rice Tinawen. *L. multiflorum* and *O. ridleyi* have been used as feeder cells to support cell division and callus growth in protoplasts of both indica and japonica rice varieties (Torrizo and Zapata, 1992: Jain et al., 1995, Lee et al., 1997b). In our experiments, *L. multiflorum* cells provided a better nurse culture for javanica rice protoplasts than the cells of *O. ridleyi*. This is in contrast to our findings with the indica varieties Pusa Basmati 1 and Jaya, for which the presence of both nurse cultures gave the highest frequency of regeneration (Jain et al., 1995). The partial replacement of sucrose with maltose as the carbohydrate source in regeneration medium and dehydration treatment of protoplast-derived colonies were both highly beneficial for induction of somatic embryogenesis and plant regeneration in the protoplast-derived tissues of javanica rice. Maltose has been also reported to be better compared to sucrose for plant regeneration in both indica and japonica rice protoplast culture (Ghosh Biswas and Zapata, 1993: Jain et al., 1995: Lee et al., 1997b). Our previous report demonstrated that dehydration treatment of protoplast-derived colonies of indica rice using a high concentration of agarose significantly enhanced plant regeneration frequency (Lee et al., 1997c). In the present study, protoplast-derived colonies of the javanica rice variey Tinawen were dehydrated in plant regeneration medium by the same procedure as described by Lee et al. (1997c). Plant regeneration frequency also increased up to 31.7%. Agarose with its solidifying effect on the medium is likely to limit water uptake by tissues, making them more compact and drier (Lai and Liu, 1988). Partial dehydration, resulting from an increased concentration of osmoticum (mannitol or sorbitol), solidifying agent (agar or agarose) or a brief plasmolysis treatment have been reported to significantly improve the frequency as well as degree of somatic embryogenesis in rice cell cultures (Lai and Liu, 1988: Tsukahara and Hirosawa, 1992: Rance et al., 1994). It is not clear how partial dehydration improves the morphogenetic potential of protoplast-derived tissues. However, the present results suggest that the osmotic pressure of the medium could be a very important factor to induce somatic embryogenesis in plant cell cultures.

적 요

Southeast Asian javanica 벼 품종 Tinawen의 진탕 배양세포로 부터 나출된 원형질체의 효과적인 배양과 식물체 재분화가 조사되었다. *Lolium multiflorum*과 *Oryza ridleyi*의 진탕 배양세포들을 feeder cell로 사용했고 여러가지 재분화 배지를 이용하여 원형질체로부터 유도된 colony들을 재분화시켰으며, 또한 식물체 재분화율을 높히기 위해 원형질체로부터 유도된 colony들을 dehydration 시켜 재분화율을 조사하였다. *L. multiflorum* 또는 *O. ridleyi*의 진탕 배양세포들을 feeder cell로 사용했을때 원형질체의 평판효율은 feeder cell type과 age에 따라 차이가 났지만 0.09%에서 1.48% 범위로

나타났고, *L. multiflorum*을 feeder cell로 사용했을때가 *O. ridleyi* cell을 사용했을때 보다 6배 높게 원형질체 평판효율을 얻었다. Feeder cell로 *L. multiflorum*을 사용하여 배양된 원형질체로부터 유도된 colony들을 dehydration 시킨 경우는 19.3-31.7%, *O. ridleyi*을 사용한 경우는 13.0-18.0%, 또한 이들 두 진탕 배양세포들을 혼합한 것을 사용한 경우는 18.0-22.0%의 식물체 재분화율을 얻은 반면에, dehydration을 시키지 않았을때는 각각 2.0-7.0%, 3.0-5.0%, 0-4.0%의 재분화율을 얻었다. 원형질체에서 재분화된 식물체의 flow cytometry를 이용한 배수성 분석 결과 대부분의 식물체가 이배체로 나타난 반면, 단지 34개중 두 식물체에서만이 4배체로 나타났다. 재분화된 식물체들은 온실에 옮겨 기른 결과 정상적인 임성을 나타내었다.

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