Theor Appl Genet (1986) 73:16–19

Haploid and diploid plant regeneration from protoplasts of anther callus in rice

Kinya Toriyama, Kokichi Hinata and Takehiko Sasaki *

Faculty of Agriculture, Tohoku University, Sendai 980, Japan

Received June 10, 1986; Accepted June 29, 1986
Communicated by G. Wenzel

Summary. The regeneration of haploid and diploid plants was demonstrated from protoplasts that were isolated from cell suspensions of anther callus in rice. The cell suspension in the AA medium that contained 4 amino acids as the sole nitrogen source was friable, finely dispersed, and readily released a large number of protoplasts. These protoplasts, subsequently cultured in NO_3 medium that contained nitrate as the sole nitrogen source, formed compact calli. The compact calli produced green plants with a frequency of 24%. Out of 15 flowering plants, 4 were haploids, the others were diploids which showed a uniform morphology but varied in seed fertility from 95 to 0%.

Key words: Anther culture – Protoplast – Regeneration – Haploid – Rice (*Oryza sativa* L.)

Introduction

Plant regeneration from protoplasts is one of the prerequisites for the application of somatic cell genetics to crop improvement. In the family Gramineae, however, which includes many economically important crops, the regeneration of plants from protoplasts has been reported for only a very few species (Vasil 1983). In rice, the regeneration of diploid plants from protoplasts has been reported only recently: Fujimura et al. (1985) and Yamada et al. (1985) demonstrated it by using the calli of seed scutellum and immature embryos, and Hayashi et al. (1986) reported successfully using the agarose bead nurse method for the protoplast culture of seed callus.

In the present study, we will discuss haploid and diploid plant regeneration from protoplasts isolated from anther calli and a viable system for the culture of totipotent haploid protoplasts in rice. The system is composed of three steps: (1) release of protoplasts from anther-derived callus in AA medium, (2) protoplast culture in NO_3 medium, and (3) plant regeneration on N6 medium.

Materials and methods

The anther-derived callus of rice (*Oryza sativa* L. cv. 'Yamahoushi') was used. Three different sources of anther calli were prepared for cell suspensions. The cell lines of YA-F and YA-3 were initiated from conventional anther culture on N6 agar medium with 2 mg/l 2,4-D (Chu et al. 1975) and AA agar medium, respectively. The cell line YP-31 was induced by "panicle culture", in which intact panicles were innoculated in AA liquid medium and calli were obtained directly from anthers in florets (Toriyama and Hinata 1985 b). These calli were subcultured at a 1:4 dilution every two weeks as cell suspensions in liquid AA medium (Toriyama and Hinata 1985 a).

The media used for protoplast culture were as follows. The B5 medium defined by Gamborg et al. (1968) was taken as a standard. In the B5-3 medium, 20 g/l sucrose, 1 mg/l 2,4-D, 0.2 mg/l kinetin and 0.1 mg/l gibberellic acid (GA_3) were added to the basic B5 medium. NO_3 medium consisted of B5-3 medium without $(NH_4)_2SO_4$, i.e. it contained nitrate as the sole nitrogen source. AA medium contained 876 mg/l glutamine, 266 mg/l aspartic acid, 174 mg/l arginine, 7.5 mg/l glycine and 2.95 g/l KCl instead of KNO_3 and $(NH_4)_2SO_4$ in B5-3 medium, i.e. it contained four amino acids as the sole nitrogen source (Müller and Grafe 1978; Toriyama and Hinata 1985 a).

Abbreviations: 2,4-D = 2,4-dichlorophenoxyacetic acid; IAA = indole-3-acetic acid

* Miyagi Prefectural Furukawa Agricultural Experiment Station, Furukawa, Miyagi 989-61, Japan

Protoplasts were isolated from the cell suspension and cultured in AA, B5-3 or NO_3 liquid medium for about 1 month. Transfer of cells from B5-3 to AA medium (B5 → AA) was also tested. Details of these methods have been described by Toriyama and Hinata (1985a). Calli derived from protoplasts were transferred to the respective agar medium or N6 agar medium supplemented with 2 mg/l 2,4-D, 1 mg/l kinetin and 3% sucrose (DK medium) for further growth.

For organogenesis, calli 1 to 2 mm in diameter were transferred to the N6 regeneration medium containing 1 mg/l kinetin, 0.2 mg/l IAA and 5% sucrose (Sasaki 1984). The regenerated plantlets were transferred again to hormone free MS medium (Murashige and Skoog 1962) or N6 medium, and then transplanted to soil in pots. They were grown in a greenhouse at Furukawa Agricultural Experiment Station in winter, and seed fertility and ploidy level were examined.

Chromosome number was determined by the method of Nishibayashi (1985) with some modifications. The root tips of regenerated plants were incubated in a 0.05 M Na-acetate buffer (pH 4.0) containing 6% Cellulase Onozuka R10, 1% Pectolyase Y23, 7.5 mM KCl and 7.5 mM EDTA at 37 °C. After a 1 h incubation. the root tips were rinsed in water several times. They were fixed with acetic alcohol (1 : 3) and then stained with 2% aceto-orcein.

Results and discussion

A finely dispersed cell suspension containing small cell clusters was obtained in the AA medium, and abundant protoplasts were readily isolated as reported previously (Toriyama and Hinata 1985a, b). The callus in the AA medium was considered to be a favorite source of protoplasts, although the reason for this has yet to be determined.

These protoplasts, when cultured in a proper medium (Table 1), showed further cell divisions and formed cell clusters (Fig. 1). The appropriate medium varied depending on the cell line, although all originated from the same cultivar. The YA-3 cell line has shown high plating efficiency in every medium so far tested. The deleterious effects of the amino acids and ammonium ion reported previously (Toriyama and Hinata 1985a) were not necessarily observed in this experiment.

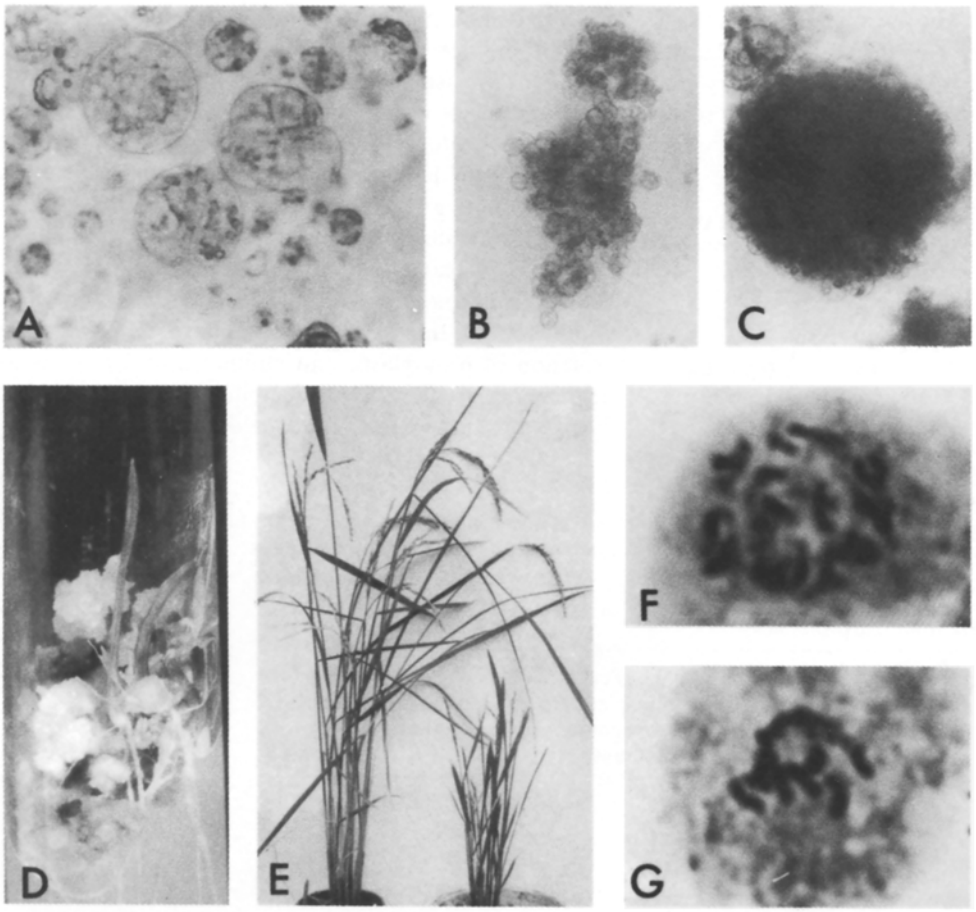

Fig. 1. **A** Protoplasts of rice after 6 days of culture. First and second divisions were observed (×400). **B** Protoplast-derived friable callus in AA medium (×100). **C** Protoplast-derived compact callus in NO_3 medium (×100). **D** Plantlet regeneration from protoplast-derived callus. **E** Diploid (*left*) and haploid (*right*) plants regenerated from protoplasts. **F** Metaphase chromosomes of diploid (2n = 24). **G** Metaphase chromosomes of haploid (n = 12)

The callus obtained from protoplasts varied in morphology according to the culture media. The callus on the NO_3 medium was compact, while that of the AA medium was very friable. Regeneration of plants was achieved when the protoplast-derived calli on the NO_3 medium were transplanted onto the regeneration medium (Fig. 1, Table 2). The calli cultured on the DK medium became very compact and also regenerated plants. On the other hand, no green spots or roots were formed in the calli cultured in the AA medium. Compact calli seemed to be superior for organogenesis to the friable ones. The regeneration frequency of the YA-F was 24% to the total callus innoculated on the regeneration medium. When the 13-passage-old cell suspension was used, the regeneration frequency was about 12%. The 6-month-old cells still retained regenerating ability.

Out of the 17 plants which originated from the 1-passage YA-F cell line, 15 plants came into flower (Fig. 1). Of these, 4 were determined to be haploid (n = 12) and 8 were diploid. The other 3 plants were suspected of being diploid in view of the size of the glumes, although the exact chromosome number has not yet been determined. Out of the 8 diploid plants, one was fully fertile having 95% seed fertility, 2 were semi-fertile (ca. 60%) and the other 5 showed low seed fertility (0–30%). No morphological variation other than seed fertility was observed among the diploid plants. All the haploid plants also showed very uniform morphology.

The presence of diploids among the regenerated plants was considered to be due to spontaneous chromosome doubling during the callus culture, since many diploids are generally observed in conventional anther cultures (Sasaki 1984). However, further investigation is necessary to determine what the source of diploids is and when the doubling of chromosomes takes place during the cultures.

Cultivation of the totipotent haploid protoplasts demonstrated in the present experiment will be utilized for somatic cell genetics such as DNA transfer, protoplast fusion, etc. The haploid protoplasts may prove valuable for these cell manipulations in comparison with the diploids because the expression of the incorporated genes could be detected easily in the haploid state and chromosomal recombination would take place more frequently than in the amphidiploid state.

The system for haploid and diploid plant regeneration from rice protoplasts is described as follows (Fig. 2): (1) Suspension culture of anther callus in the AA medium produces friable calli that readily release abundant protoplasts. Panicle culture proposed by Toriyama and Hinata (1985b) may also be available. (2) High plating efficiency of protoplasts is obtained in the NO_3 medium and the calli become compact. In addition, (3) plant regeneration can be obtained on the N6 regeneration medium. In this system, it takes 2 to 3 months from the initiation of anther culture to the isolation of protoplasts, and another 2 to 3 months to regenerate plantlets. Reproducibility of this system has been confirmed in other cultivars of rice.

Table 1. Plating efficiency in culture media. Number of colonies expressed as a percentage of the total protoplast population (− = 0%, + = > 0.01%, + + = > 0.1%, + + + = > 1%)

Cell line	Plating efficiency (%)			
	NO_3	B5-3	AA	B5 → AA[a]
YA-F	+ + +	+	−	+
YA-3	+ + +	+ + +	+ + +	+ +
YP-31	+	+	+ +	−

[a] After initial culture in B5-3 medium for 8–10 days, the cells were transferred to AA medium

Acknowledgements. We are grateful to Mr. N. Konno for his assistance in observing chromosomes.

Table 2. Regeneration frequency from rice protoplasts

Cell line	Passage no.	Liquid media for protoplast culture	Agar media for further growth	No. of callus transplanted to regeneration medium	No. of callus produced plantlets	
					Green	Albino
YA-F	1	NO_3	NO_3	70	17	1
YA-F	13	NO_3	NO_3	15	1	0
		NO_3	DK	10	2	0
YA-3	3	NO_3	DK	10	3	1
		B5-3	DK	10	1	0
		AA	DK	10	0	0
		B5 → AA	DK	10	1	1
YP-31	5	NO_3	DK	15	0	3

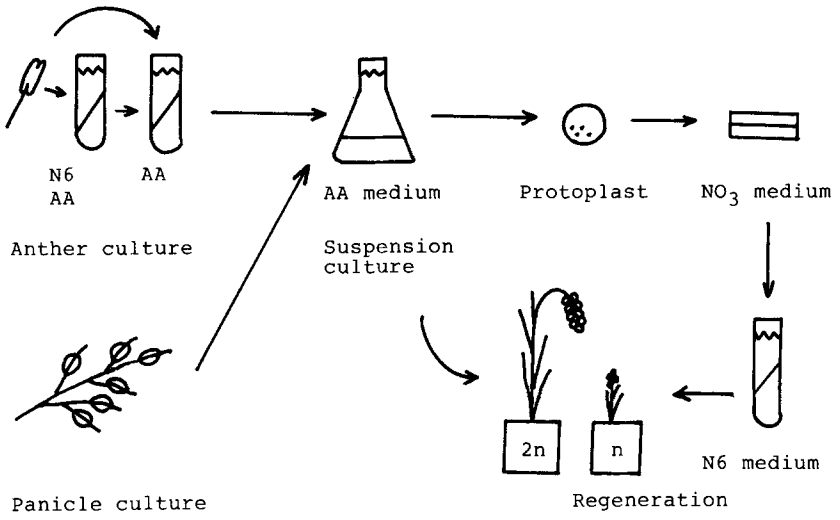

Fig. 2. A scheme for plant regeneration from protoplasts of anther calli in rice

References

Chu CC, Wang CC, Sun CS, Hsu C, Yin KC, Chu CY, Bi FY (1975) Establishment of an efficient medium for anther culture of rice through comparative experiments on the nitrogen sources. Sci Sin 18:659–668

Fujimura T, Sakurai M, Akagi H, Negishi T, Hirose A (1985) Regeneration of rice plants from protoplasts. Plant Tissue Culture Lett 2:74–75

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50:151–158

Hayashi Y, Kyozuka J, Shimamoto K (1986) Plant regeneration from protoplasts of rice (*Oryza sativa*). Jpn J Breed 36 (Suppl 1): 46–47

Müller AJ, Grafe R (1978) Isolation and characterization of cell lines of *Nicotiana tabacum* lacking nitrate reductase. Mol Gen Genet 161:67–76

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Nishibayashi S (1985) Chromosomal analysis in rice. Conditions to observe chromosomes. Jpn J Breed 35 (Suppl 1): 318–319

Sasaki T (1984) Application of anther culture in rice breeding. 1. Present status and problems of culture technique. Rep Tohoku Br Crop Sci Soc Jpn 27:75–78

Toriyama K, Hinata K (1985a) Cell suspension and protoplast culture in rice. Plant Sci 41:179–183

Toriyama K, Hinata K (1985b) Panicle culture in liquid media for obtaining anther calli and protoplasts in rice. Jpn J Breed 35:449–452

Vasil IK (1983) Isolation and culture of protoplasts of grasses. Int Rev Cytol (Suppl) 16:79–88

Yamada Y, Yang ZQ, Tang DT (1985) Regeneration of rice plants from protoplasts. Rice Genet Newslett 2:94–95