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Plant regeneration from protoplasts of indica rice: genotypic differences in culture response

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Summary. Fourteen varieties of indica rice (*Oryza sativa* L.) were examined for their capacity for plant regeneration from protoplasts using the nurse culture methods developed for japonica rice. Calli induced from germinating seeds were grouped into two types: type I, white and compact; type II, yellow and friable. In four varieties producing type II callus, colony formation (2%–4.5%) and plant regeneration (2%–35%) were observed. The inability to develop suspension cultures was a major obstacle in regenerating plants from protoplasts of the remaining rice varieties studied.

Key words: Rice (*Oryza sativa* L.) – Cereals – Protoplast – Indica rice – Plant regeneration

Introduction

Plant regeneration from rice protoplasts has recently been reported by several groups (Fujimura et al. 1985; Abdullah et al. 1986; Toriyama et al. 1986; Yamada et al. 1986; Kyozuka et al. 1987). However, the protoplasts employed in all of these studies were isolated from japonica varieties of rice. Cultivated rice (*Oryza sativa* L.) is divided into three subspecies – indica, japonica, and javanica – and the indica varieties are the most widely grown, covering temperate to tropical regions and occupying the largest portion of the cultivated rice area in the world. Hence, in order to use protoplast regeneration as a tool in rice improvement, reproducible methods for protoplast culture of indica rice varieties need to be established.

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In an earlier report, we described high frequency plant regeneration from protoplasts of four japonica varieties using the newly developed nurse culture methods (Kyozuka et al. 1987). More recently, somatic hybrids of rice and barnyard grass (Terada et al. 1987) and of rice and wild *Oryza* species (Hayashi et al. 1988) have been obtained using electrofusion and the nurse culture method. Field performances of protoplast-derived rice plants suggest that protoplast culture can be a valuable tool in rice improvement (Ogura et al. 1987). In this paper, we describe plant regeneration from protoplasts of four indica rice varieties. We also show that the response to culture conditions varies greatly among indica varieties and suggest possibilities for improving plant regeneration from protoplasts of recalcitrant varieties.

Materials and methods

Initiation of suspension cultures

Seeds of 'Boro 8' were kindly supplied by Dr. Osada of Utsunomiya University, Utsunomiya, and 'IR 3646', 'Lawng Tawng', 'Kinangdang Puti', '63–83', and 'IRAT 109' were supplied by the Ibaraki Prefecture Agriculture Center (Mito). The rest of the seed materials were obtained from the Institute of Agrobiological Resources, Tsukuba. All seeds were surface sterilized in 0.12% sodium hypochlorite for 60 min, followed by two rinses with sterile distilled water. Sterilized seeds were inoculated on MS medium (Murashige and Skoog 1962) with 3% sucrose, 2 mg/l 2,4-D, and 0.8% agarose (type I, Sigma) under light. Three to four weeks later, callus was transferred to flasks containing liquid R2 medium (Ohira et al. 1973) supplemented with 3% sucrose and 2 mg/l 2,4-D in order to establish suspension cultures. Liquid cultures were maintained by weekly subculture and were shaken at low speed (approximately 60 rpm).

Protoplast isolation and culture

One month-old suspension cultures were used for protoplast isolation. Protoplasts were isolated from suspension cultures

3–5 days after subculture. Cultured cells were placed in a 10-cm plastic plate containing 20 ml of an enzyme mixture consisting of 4% Cellulase RS (Kinki Yakult, Japan), 1% Macerozyme R10 (Kinki Yakult, Japan) and 0.4 M mannitol (ph 5.6) without shaking at 30 °C for 3–4 h. The resulting suspension was then filtered through a 20-μm nylon mesh and 4 × volume of KMC solution (Harms and Potrykus 1978) was added. After centrifugation for 8 min at 800 rpm, pelleted protoplasts were collected and washed twice in the KMC solution.

Protoplasts were cultured by the mixed nurse culture method previously reported (Kyozuka et al. 1987). Purified protoplasts were plated at a density of 5×10^5/ml in R2 medium containing 0.4 M sucrose, 2 mg/l 2,4-D, and solidified with 1.2% Sea Plaque agarose (FMC, USA). Solidified agarose medium containing the protoplasts was cut into blocks, which were then placed into 5 ml liquid R2 protoplast medium (Kyozuka et al. 1987) containing approximately 100 mg of nurse cells.

Suspension cultures of rice Oc cells (Baba et al. 1986) were used as nurse cells. Plates were shaken slowly (20 rpm) in the dark at 27 °C. After 10 days of culture, agarose blocks were transferred to new plates and Oc cells were removed completely by washing 2–3 times with the culture medium. After 3 weeks of culture, agarose blocks containing visible colonies were transferred onto N6 (Chu et al. 1975) soft agar medium supplemented with 2 mg/l 2,4-D, 6% sucrose, and 0.25% agarose. Two weeks later, colonies 1 mm in diameter were individually transferred to the same medium, but with a higher agarose concentration (0.5%). When the colonies were about 2 mm in diameter, they were transferred to regeneration medium containing N6 basal components, 1% agarose, and 6% sucrose (N6–6S), or N6 basal components, 1% agarose, 3% sucrose, and 2% sorbitol (N6–SS) (Kavi-Kishjor and Reddy 1986). After the regenerated shoots attained a length of 2 cm or more, they were transferred to plastic boxes containing 30 ml N6 regeneration medium with 0.8% agarose.

Results

Initiation of suspension culture

Calli were induced from mature seeds of 14 indica varieties of rice. Using the criteria adapted for distinguishing the two types of maize calli (Green and Phillips 1975; Lu et al. 1982; Armstrong and Green 1985), the rice calli were grouped into two types on the basis of morphology and appearance. Callus of some varieties was yellow, relatively friable, and embryogenic (type II), similar to callus of japonica varieties; other varieties formed white and compact callus (type I), from which suspension cultures were difficult or impossible to develop; still other varieties produced a mixture of type I and type II calli. While suspension cultures could be readily developed from type II callus within 1 month, type I callus turned brown immediately upon transfer to liquid medium. Suspension cultures were successfully developed from six indica rice varieties (Table 1). Appearances and growth rates of these suspension cultures were indistinguishable from those of standard japonica rice varieties (eg. 'Nipponbare').

Table 1. Genotypic effect on culture response among indica varieties of rice

Variety	Callus initiation	Callus type[a]	Suspension culture	Protoplast culture	Shoot regeneration
IR 8	+	I	–	–	–
IR 24	+	I	–	–	–
IR 26	+	I	–	–	–
IR 36	+	I	–	–	–
IR 3646	+	I	–	–	–
Chinsurah Boro II	+	I, II	+	+	+
Boro 8	+	I, II	+	NT	NT
63-83	+	II	+	+	+
IRAT 109	+	II	+	+	+
Cyokoto	+	II	+	+	+
Padma	+	I	–	–	–
Ratna	+	I	–	–	–
Lawng Tawng	+	I	–	–	–
Kinangdang Puti	+	II	+	+	NT

NT: not tested
[a] Callus type I: white, compact; type II: yellow, friable

Table 2. Plating efficiencies and shoot regeneration frequencies from protoplasts of indica rice varieties

Variety	Plating efficiency (%)[a]	Regeneration medium	Shoot regeneration frequency (%)
Chinsurah Boro II	NT	N6-6S	34.6
63-83	2.1	N6-6S	25.9
IRAT 109	2.0	N6-6S	14.8
Chyokoto	4.5	N6-SS	2.0
Kinangdang Puti	2.1	NT	NT

NT: not tested
[a] Plating efficiency was obtained after 12 days of culture

Protoplast culture

Protoplast isolation was possible after 3–4 weeks of culture in R2 liquid medium. Protoplasts were isolated 3–5 days afte subculture, and average yields of $4-6 \times 10^6$ protoplasts/g. F.W. were obtained. The isolated protoplasts were small (10–20 μm in diameter), cytoplasmically rich (Fig. 1 a), and relatively free from contamination with cell debris or undigested cells. They were cultured by a mixed nurse culture method. First divisions were observed on day 3–5, and small colonies formed within 2 weeks.

The protoplast-derived colonies of 'Kinangdang Puti', '63–83', 'Chinsurah Boro II' and 'IRAT 109' were compact and consisted of small cytoplasmically rich cells, whereas 'Cyokoto' callus was rather friable. Plating efficiencies observed on day 12 were 2%–5% (Table 2).

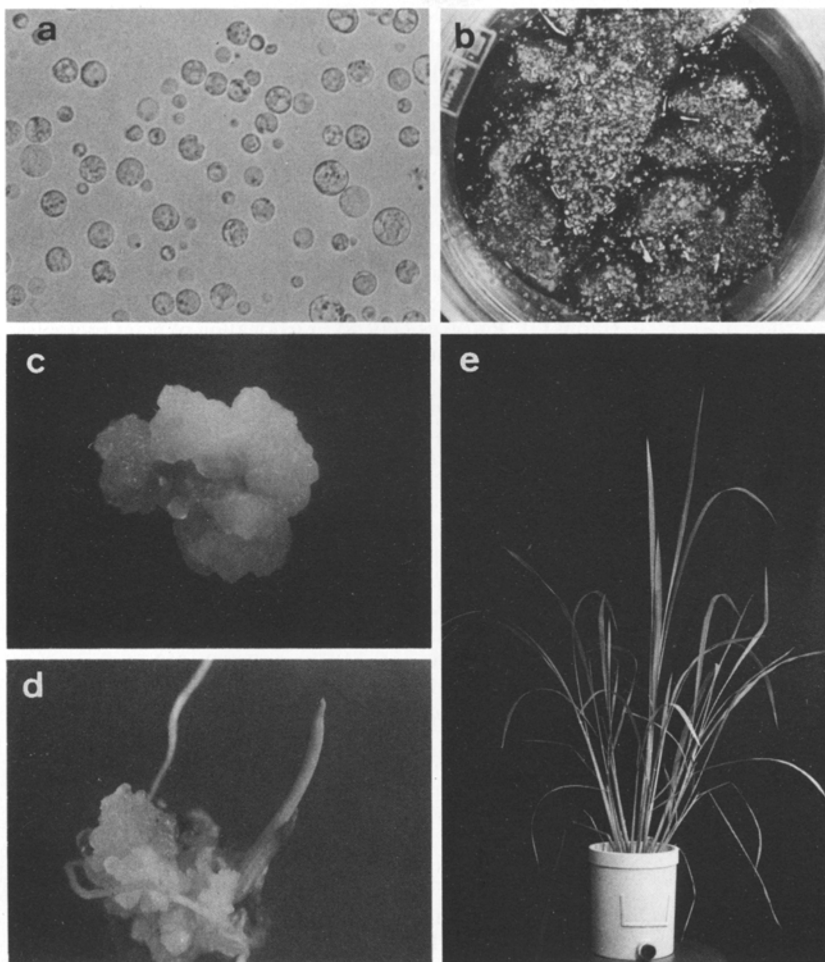

Fig. 1 a–e. Protoplast culture and plant regeneration from indica rice. **a** Freshly isolated protoplasts (cv '63–83'); **b** colonies growing on N6 soft agar medium (cv 'Chyokoto'); **c** embryogenic callus derived from protoplasts (cv '63–83'); **d** Shoot formation from protoplast-derived callus on N6-SS regeneration medium (cv '63–83'); **e** regenerated plant 4 weeks after transfer to soil (cv 'Chyokoto')

Colonies were visible after 3 weeks of culture, at which time agarose blocks with numerous colonies were transferred to N6 soft agar medium for further growth (Fig. 1 b). After 7–10 days on the soft agar medium, colonies were individually transferred to the same medium but with a higher agarose concentration for stimulating growth.

Plant regeneration

Callus became compact and embryogenic within 2–3 weeks after transfer to the N6 regeneration medium (Fig. 1 c). Shoots formed 2–7 weeks later (Fig. 1 d). The frequencies of shoot formation varied among varieties and were higher than 20% for '63–83' and 'Chinsurah Boro II', and 2% for 'Cyokoto' (Table 2). Occasionally, well-developed roots appeared prior to shoot formation. Embryo-like structures were observed on protoplast-derived callus. However, some of these embryos did not continue to grow, and only about half developed into plants. Regenerated plants appeared morphologically normal, and albino plants were not observed (Fig. 1 e).

Discussion

Indica varieties varied in their response to culture, some responding as well as japonica varieties. In contrast to this variability in the response of indica varieties, little variation was found in japonica varieties. Plant regeneration has been obtained in all of the protoplasts from all japonica varieties examined thus far (Kyozuka et al. unpublished results). In our study, only those indica varieties that behaved similarly to the japonica varieties in culture gave rise to plants. It was very difficult to establish suspension cultures of non-responding indica varieties, which produced type I callus. Callus of this type often turned brown in liquid medium. However, once suspension cultures were established, protoplast isolation, colony formation, and shoot regeneration could be accomplished by use of the nurse culture method. Plants were also regenerated from protoplasts of a javanica rice, 'IR 279'. From this variety, suspension cultures were easily obtained by the procedure described above, and the plating efficiency and plant regeneration frequency were 4.1% and 21.0%, respectively.

One of the important medium constituents influencing the ability of cells to be cultured is the nitrogen source. The nitrogen source has been found to be important for the development of cell suspension cultures (Thompson et al. 1986) and for sustained division of protoplasts (Toriyama and Hinata 1985) of rice. Our preliminary results show that, as reported by Thompson et al. (1986), AA medium (Müller and Grafe 1978), which contains amino acids as the sole nitrogen source, was effective in reducing the browning of the suspension cultures.

Protoplasts isolated directly from primary callus of japonica varieties form colonies and regenerate plants (Kyozuka et al. 1987). In indica varieties, a large number of protoplasts could be isolated from callus that had been subcultured once or twice on MS callus induction medium (data not shown). Although their plating efficiencies were very low, these protoplasts could be used in cell fusion experiments.

The plating efficiencies obtained with indica protoplasts in this study were comparable to those, obtained from japonica rice varieties (Kyozuka et al. 1987). However, the frequency of plant regeneration from protoplast-derived callus of indica varieties was lower than the plant regeneration frequency from protoplasts of japonica rice varieties. Growth of regenerating indica shoots occasionally stopped at the plantlet stage, a phenomenon not common in japonica varieties. Although protoplast-derived plant could be obtained from indica rice varieties, the genotype dependence of this success remains a problem. Studies on developing general culture procedures for indica rice varieties are in progress.

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