Theor Appl Genet (1987) 74:670–676

Field performance and cytology of protoplast-derived rice (*Oryza sativa*): high yield and low degree of variation of four japonica cultivars*

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Received March 27, 1987; Accepted March 27, 1987
Communicated by I. Potrykus

Summary. Protoplast-derived rice plants of four Japanese cultivars, Nipponbare, Fujisaka 5, Norin 14 and Iwaimochi were individually cultivated in a submerged paddy field. They exhibited more stems, which resulted in more panicles than respective control plants. Other characteristics of protoplast-derived plants were (compared with controls): a slightly shorter or similar culm length, fewer spikelets per panicle, slightly lower seed fertility and similar or lighter 1,000 kernel weight. Grain yield of protoplast-derived plants was more than that of respective control plants in four cultivars. The cause of the higher yield of protoplast-derived plants seems to be mainly due to increased panicle number. Among 126 protoplast-derived plants, 1 triploid, 10 tetraploids and 1 aneuploid were found. Furthermore, 11 variants with low seed fertility showing no gross chromosomal anomalies and one plant with abnormal panicles were found. In total, about 80% of protoplast-derived plants showed normal characters. The present results are encouraging for the possibility of rice breeding using protoplasts.

Key words: Rice cultivars – Protoplast-derived plants – Agronomic characters – Cytology – Somaclonal variation

Introduction

Despite the progress in genetic manipulation of dicotyledonous species, studies on transformation or somatic hybridization in *Gramineae* (Potrykus et al. 1985; Lörz et al. 1985; Fromm et al. 1986; Uchimiya et al. 1986; Baba et al. 1986; Ozias-Akins et al. 1986; Tabaeizadeh et al. 1986) have been all performed at the level of cultured cells due to the lack of a reproducible method from protoplasts. Several recent reports, however, concern plant regeneration from rice protoplasts (Fujimura et al. 1985; Hayashi et al. 1986; Yamada et al. 1986; Coulibaly and Demarly 1986; Abdullah et al. 1986). It is expected, therefore, that genetically engineered rice plants using protoplasts will be obtained in the near future. In such cases, characterization and cytogenetical analysis of protoplast-derived plants will become inevitable.

In dicotyledonous species, characterization or cytological analysis of protoplast-derived plants has been reported in tobacco (Lörz and Snowcroft 1983), potato (Shepard et al. 1980; Karp et al. 1982; Sree Ramulu et al. 1983; Gill et al. 1985) and alfalfa (Johnson et al. 1984). In monocotyledonous species, including *Gramineae,* no such studies have been reported so far.

Recently we developed a high frequency (up to 50%) plant regeneration system from rice protoplasts by novel nurse culture methods (Kyozuka et al. 1987). Protoplast-derived plants of four rice cultivars obtained by this system were cultivated in the submerged paddy field. Here, we report the results of character investigation and cytological analysis of protoplast-derived plants.

Materials and methods

Protoplast source, isolation, culture and plant regeneration

Calli were initiated from mature seeds or immature embryos of four cultivars of rice: Nipponbare, Fujisaka 5, Norin 14 and

* This paper is dedicated to Professor Emeritus Dr. Shigeyasu Akai on the occasion of his 77th birthday (Kijyu: one of the celebrated ages in Japan)

Iwaimochi. Seeds of Fujisaka 5, Norin 14 and Iwaimochi were obtained from the Institute of Agrobiological Resources, Tsukuba. Nipponbare, Fujisaka 5 and Norin 14 are starchy cultivars, whereas Iwaimochi is waxy. Protoplasts were isolated from seed calli or suspension cultures of embryo origin. Protoplasts were cultured by new nurse culture methods (Hayashi et al. 1986). Regeneration medium consisted of N6 basal medium (Chu et al. 1975), 6% sucrose, 1% agarose and various concentrations of cytokinins. Within 3–8 weeks, shoots and roots were visible and when shoots became 3 cm or longer they were transferred to a plastic container containing 50 ml of hormone-free N6 regeneration medium with 0.8% agarose. Detailed methods are described in Kyozuka et al. (1987). When protoplast-derived plantlets in a plastic container became 5–8 cm in height, they were transferred to raising plates containing sterilized soil and fertilizer on April 16, 1986 and were grown in the greenhouse. At the same time, rice seeds of each cultivar were sown in raising plates as control and reared similarly.

Transplanting in the paddy field

There were 75 seedlings of Nipponbare, 13 of Fujisaka 5, 13 of Norin 14 and 30 of Iwaimochi transferred to the submerged paddy field on May 11, 1986. They were planted individually with an inter-row space of 30 cm and hill space within a row of 30 cm. Seed-sown control seedlings were planted in the same manner, except for Norin 14. Sowing and transplanting of both protoplast-derived and the control seedlings of Norin 14 were delayed, therefore some Norin 14 data are not shown. Fertilizers and chemicals were applied according to the standard paddy field culture method of Ishikawa Prefecture.

Investigation method of growth and yield characters

There were 25 protoplast-derived plants of Nipponbare, 9 of Fujisaka 5, 13 of Norin 14 and 20 of Iwaimochi used for growth investigation. Growth was examined 7–9 times as seedling height and stem number until heading. Heading date was determined on the day when 50% of the plants of a line headed. Maturation date was determined on the day when 90% of the plants of a line ripened yellow. After maturation, each plant was carefully harvested with its root by digging the soil. After drying in the greenhouse, several characters were investigated. Culm length, panicle length, spikelet number and filled spikelet number were measured using the longest stem of each plant. After the measurement, yield data were collected using two representative plants in each cultivar.

Cytology

Young root-tips of several seedlings in each cultivar were collected before transplanting in the paddy field. They were pretreated with saturated 8-hydroxyquinoline solution (0.002 M) for 2–2.5 h at 18 °C and fixed with acetic alcohol solution (1:3). They were squashed with 1% acetocarmine solution and the slide was dipped in liquid nitrogen, then the cover glass was removed with a razor. The slide was dipped in 1.5% Giemsa solution (Giemsa original solution was diluted with 1/15 M phosphate buffer, pH 6.8) for 20–40 min, then washed gently in distilled water. After air-drying, the slide was used for microscopic observation. In some cases, the flame-drying method (Kurata and Omura 1978) was used. For observation of meiosis, well-developed stems sheathing young panicles were collected and one anther of each spikelet was directly squashed with 1% acetocarmine solution and examined under a microscope. If the anther contained PMC's of a suitable meiotic stage, then the other five anthers were fixed with acetic alcohol (1:3). After fixation, the same procedure was applied to anthers as to root-tips.

Results

Growth habit

The changes in plant height and stem number of three cultivars after transplanting of rice plants are shown in Tables 1 and 2. At the time of rice transplanting in the paddy field, protoplast-derived seedlings of each cultivar were mostly higher than controls, and few had one or two tillers. However, at the final investigation, plant heights of both the protoplast-derived and the control plants of Nipponbare and Iwaimochi were similar. In Fujisaka 5, protoplast-derived plants were significantly lower than control plants. Stem number of protoplast-derived plants were significantly increased in three cultivars. Protoplast-derived plants cultivated in the paddy field are seen in Fig. 1 a.

Heading and maturation date

As depicted in Tables 3–5, heading date did not greatly differ between protoplast-derived and control plants in

Table 1. Changes of plant height (cm ± SE) of protoplast-derived plants of three cultivars

Line[a]	No. of plants	Date of measurement								
		10/5	5/6	14/6	25/6	3/7	12/7	21/7	30/7	8/8
Nipponbare (Pt)	25	9.4±0.7	23.5±0.9	27.5±0.4	38.1±0.8	47.8±0.8	52.8±1.1	63.0±0.7	70.6±1.0	76.7±1.0
Nipponbare (Contr)	10	8.4±0.4	28.8±0.6	33.5±0.4	42.6±0.8	52.5±0.8	58.4±0.8	64.6±0.7	70.8±1.0	75.2±1.2
Fujisaka 5 (Pt)	9	14.7±1.1	29.9±1.6	34.4±1.7	48.2±1.2	59.9±1.5	65.5±1.6	73.8±1.2	78.8±1.3	–
Fujisaka 5 (Contr)	10	9.7±0.4	35.3±0.8	40.7±0.8	59.0±1.1	67.8±0.9	74.3±1.2	85.5±1.4	92.4±1.5	–
Iwaimochi (Pt)	20	15.1±1.0	26.8±0.9	30.0±0.9	40.0±0.9	50.1±1.1	57.9±1.2	67.8±1.1	75.8±1.2	80.2±1.2
Iwaimochi (Contr)	10	7.9±0.4	25.5±0.6	29.8±0.3	40.5±1.3	50.6±0.8	56.5±1.5	68.8±1.7	77.0±1.5	81.4±1.6

[a] Pt: protoplast-derived plants; Contr: control

Table 2. Changes of stem number (average number per plant ± SE) of protoplast-derived plants of three cultivars

Line[a]	No. of plants	Date of measurement								
		10/5	5/6	14/6	25/6	3/7	12/7	21/7	30/7	8/8
Nipponbare (Pt)	25	1.0±0.0	5.5±0.6	10.0±1.1	22.6±2.2	28.9±2.4	35.6±2.4	37.3±2.1	38.0±2.3	34.9±2.2
Nipponbare (Contr)	10	1.0±0.0	4.8±0.5	10.0±1.0	19.4±1.9	27.6±2.1	33.7±1.9	32.6±2.1	31.4±2.1	26.3±2.0
Fujisaka 5 (Pt)	9	1.2±0.2	9.5±1.4	13.9±2.1	23.7±3.3	26.1±2.8	25.4±2.5	–	24.5±2.2	–
Fujisaka 5 (Contr)	10	1.0±0.0	4.8±0.3	7.2±0.6	15.1±1.1	18.4±1.0	18.7±0.8	–	17.5±0.7	–
Iwaimochi (Pt)	20	1.5±0.1	12.7±0.9	22.2±2.0	40.9±2.8	45.0±2.9	48.5±3.0	46.7±2.8	45.4±2.8	43.4±2.7
Iwaimochi (Contr)	10	1.0±0.0	7.3±0.2	13.5±0.8	27.9±1.2	33.6±1.3	39.0±1.8	37.2±1.4	35.2±1.1	30.7±1.3

[a] Pt: protoplast-derived plants; Contr: control

Table 3. Comparison of agronomic characters between protoplast-derived plants and seed-sown control plants in a cultivar Nipponbare

	No. of plants	Heading date (range)	Maturation date	Culm length		Panicle length		Panicle no. per m²	Spikelet no. per panicle	Seed fertility (%)	1,000 grain weight (g)	Grain yield (kg/10a)
				Mean ±SE	CV (%)	Mean ±SE	CV (%)					
Protoplast-derived plants	25	Aug. 20 (11–23)	Oct. 2	68.2±0.9	6.6	18.0±0.3	7.8	388.5	74.4	91.5	20.2	534.2
Control plants	10	Aug. 19 (15–19)	Sept. 29	69.0±1.0	4.3	19.5±0.6	9.7	272.0	78.8	93.1	20.3	405.1

Table 4. Comparison of agronomic characters between protoplast-derived plants and seed-sown control plants in a cultivar Iwaimochi

	No. of plants	Heading date (range)	Maturation date	Culm length		Panicle length		Panicle no. per m²	Spikelet no. per panicle	Seed fertility (%)	1,000 grain weight (g)	Grain yield (kg/10a)
				Mean ±SE	CV (%)	Mean ±SE	CV (%)					
Protoplast-derived plants	20	Aug. 24 (14–30)	Oct. 15	75.5±1.0	6.1	20.0±0.4	9.5	577.2	67.5	53.9	18.9	396.9
Control plants	10	Aug. 27 (21–27)	Oct. 15	75.9±1.5	6.7	21.6±0.5	7.4	288.6	72.6	75.4	20.3	320.7

Table 5. Comparison of agronomic characters between protoplast-derived plants and seed-sown control plants in a cultivar Fujisaka 5

	No. of plants	Heading date (range)	Maturation date	Culm length		Panicle length		Panicle no. per m²	Spikelet no. per panicle	Seed fertility (%)	1,000 grain weight (g)	Grain yield (kg/10a)
				Mean ±SE	CV (%)	Mean ±SE	CV (%)					
Protoplast-derived plants	9	July 24 (17–28)	Aug. 30	56.2±1.2	6.6	19.4±0.7	11.3	317.5	81.4	86.4	20.8	464.5
Control plants	10	July 25 (22–30)	Sept. 1	71.9±1.2	5.4	19.2±0.4	6.8	198.7	113.0	91.6	22.1	454.5

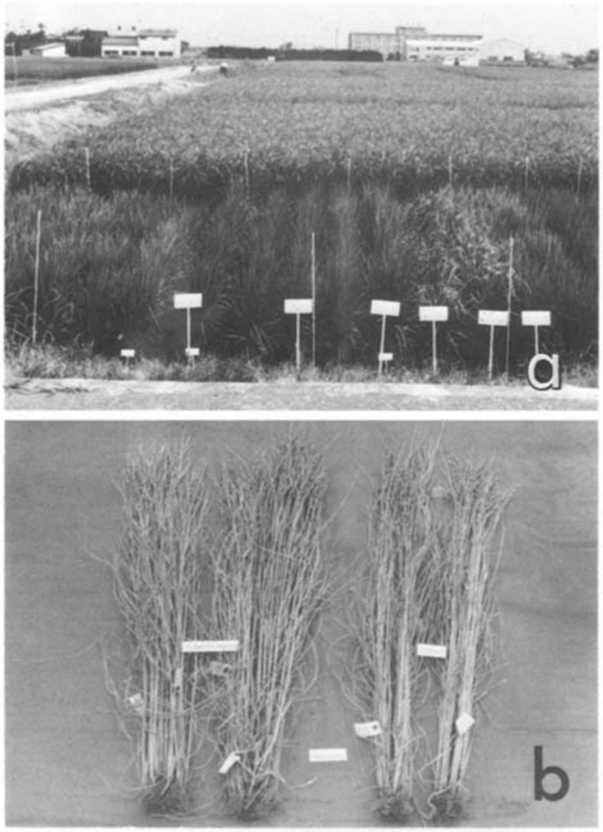

Fig. 1. **a** Protoplast-derived plants cultivated in the paddy field; **b** Dried specimens of harvested rice plants of Iwaimochi: *left*, two protoplast-derived plants with an increased number of panicles; *right*, two controls

each cultivar, however the ranges were wider in protoplast-derivatives. Maturation date was also similar between two lines in three cultivars, the ranges being also wider in protoplast-derived lines.

Culm and panicle length

Culm length of Fujisaka 5 was apparently shorter than the controls, but panicle length was similar. Culm length of Nipponbare and Iwaimochi was similar to the controls, but, panicle length was slightly shorter in protoplast-derived lines. Examples of panicles of Nipponbare and Iwaimochi are shown in Fig. 3a–d.

Yield characters

As shown in Tables 3–5, the panicle number in protoplast-derived plants was greater than controls in three cultivars; protoplast-derived Iwaimochi in particular showed far more panicles than did the control (Table 4 and Fig. 1b). However, average spikelet number per panicle was lower in protoplast-derived plants in the three cultivars. Seed fertility was a little lower in protoplast-derivatives of Nipponbare and Fujisaka 5.

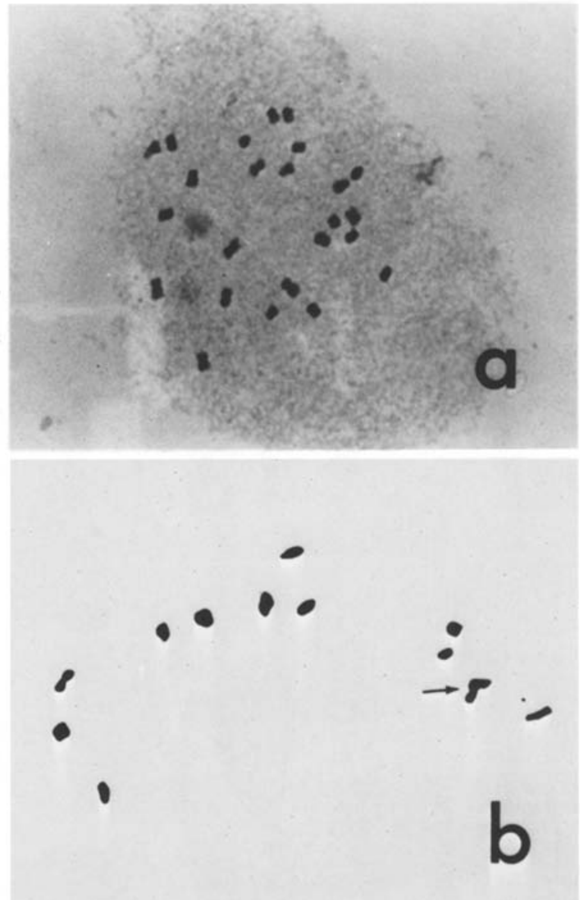

Fig. 2a, b. *Chromosomes of Nipponbare.* **a** Metaphase chromosomes of a root-tip cell of a protoplast-derived plant, showing normal $2n=24$ chromosome number; **b** Metaphase chromosome configuration of a PMC of a protoplast-derived trisomic plant, showing $1_{III}+11_{II}$; *arrow* indicates a trivalent chromosome

In Iwaimochi, percentage of fertility was very low in protoplast-derived plants, although control plants also showed a comparatively low percentage of seed fertility. 1,000 dehusked kernel weight was generally a little lower in protoplast-derived plants in the three cultivars. By summing up the data of each yield component, grain yield of protoplast-derived plants was calculated to be higher than control plants in the three cultivars.

Somaclonal variation

Most of protoplast-derived rice plants were diploids, however three tetraploids and one aneuploid were found in Nipponbare, one triploid and four tetraploids in Iwaimochi, one tetraploid in Fujisaka 5 and two tetraploids in Norin 14. Examples of somatic and meiotic chromosomes are seen in Fig. 2. Table 6 shows the number and frequency of somaclonal variants of the four cultivars. Among these, chromosomal variants

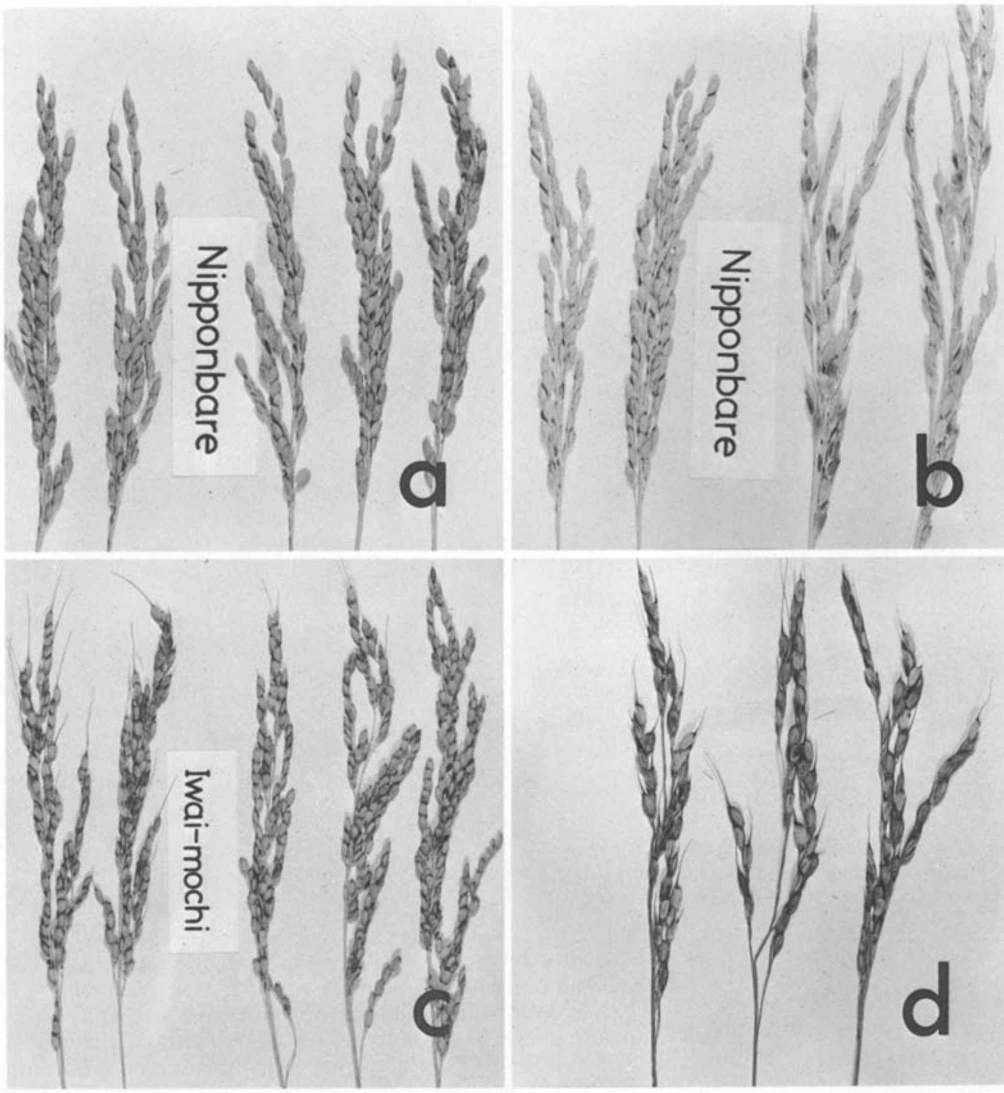

Fig. 3a–d. Panicles of protoplast-derived plants: **a** *right*, three protoplast-derived and *left*, two control Nipponbare; **b** *right*, two protoplast-derived tetraploid and *left*, two control Nipponbare; **c** *right*, three protoplast-derived and *left*, two control Iwaimochi; **d** protoplast-derived triploid of Iwaimochi

Table 6. Somaclonal variation observed in protoplast-derived plants of four rice cultivars

Cultivar	No. of plants	No. of normal plants (%)	Somaclonal variants		
			Chromosomal variants (%)	Low seed fertiliy variants (%)	Undetermined variants (%)
Nipponbare	73	61 (83.6)	4 (5.5)	8 (11.0)	
Fujisaka 5	11	9 (81.8)	1 (9.1)	1 (9.1)	
Norin 14	13	11 (84.6)	2 (15.4)		
Iwaimochi	29	21 (72.4)	5 (17.2)	2 (6.9)	1 (3.4)
Total	126	102 (81.0)	12 (9.5)	11 (8.7)	1 (0.8)

were always accompanied by abnormal phenotypes, e.g. abnormal panicles (Fig. 3b, d) and/or dwarfism. Plants with less than 40% seed fertility were classified as low seed fertility variants. They were all diploid and most had an almost normal appearance. One undetermined variant of Iwaimochi had abnormal panicles, but these were not cytologically examined.

Discussion

To our knowledge, this is the first report of the field performance of protoplast-derived rice plants. The results indicate that protoplast-derived rice plants were generally 'normal' but produced a greater grain yield. In this experiment, a total of 19% of protoplast-derivatives showed variation, however this is not as high as other results from tissue-culture-derived rice plants or protoplast-derived plants of other species. Oono (1975) reported that about one-third of anther-derived rice plants showed some anomalies. Oono (1981) also observed that 72% of 1,121 regenerated plants from rice seed callus showed some mutated morphological characters. Secor and Shepard (1981) reported 58 clones out of 65 derived from protoplasts of Russet Burbank potato exhibited differences in at least one character from the parental cultivar. Johnson et al. (1984) compared two lines of alfalfa plants with their parent clones, and found only 30% and 45% of protoclones in each had a parental chromosome number of $2n=32$.

In this experiment, the frequency (ca. 10%) of chromosomal variants does not seem to be high. Nishi and Mitsuoka (1969) reported that among 16 plants obtained through anther culture, one was triploid and seven pentaploid. Chen and Lin (1976) observed 23 tetraploids among 165 regenerated plants of 5 japonica varieties of rice. Oono (1981) identified 2 trisomics, 5 triploids and 1 tetraploid in 70 anther-derived rice plants. Recently Mercy and Zapata (1986) reported that among 209 plants derived from anther culture of rice, one was triploid, one was hypotetraploid and one was tetraploid. In the present experiment, the appearance of tetraploid plants was conspicuous among chromosomal variants. Probably chromosomal duplication due to endomitosis occurs during the culture or plant regeneration takes place from callus derived from fused protoplasts. Low seed fertility variants, which showed no obvious chromosomal variations, may be due to minute genetic changes probably during the tissue culture. Further examination of progeny plants of these variants are in progress.

'Seedling age' of protoplast-derived plants were generally higher than seed-sown controls in each cultivar. Nevertheless, there are many reports suggesting that effects of 'seedling age' on yield characters are within 5%. Thus, outlines of agronomic traits of protoplast-derived plants can be grasped by comparing the data presented in Tables 3–5.

Grain yield of protoplast-derived lines was higher than the controls in four cultivars (Norin 14 data not shown), although each grain yield among four cultivars differed considerably. The higher yield of protoplast-derived plants in each cultivar is mainly due to more panicle number. Whether this trait is heritable or not must await field testing of progeny plants of protoplast-derived plants. As a whole, protoplast-derived rice plants showed comparable grain yield and a relatively low degree of variation compared to seed-sown controls. These are encouraging results for rice breeding using protoplasts and genetically manipulated rice plants. Selfed seeds of several protoplast-derived plants exhibiting favourable phenotypes will be the subjects of further studies.

Acknowledgements. We wish to thank Dr. R. Ito of Plantech Research Institute and Prof. H. Niizeki of Ishikawa College of Agriculture for their helpful suggestions. Thanks are also due to Dr. M. Fukutomi of the College for his gift of liquid nitrogen. This work was partly supported by special research grant of Ishikawa College of Agriculture to H.O.

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