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# Diploid somatic-hybrid plants regenerated from rice cultivars

**K. Toriyama and K. Hinata**
Faculty of Agriculture, Tohoku University, Senday 980, Japan

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**Summary.** Somatic hybrid plants were obtained between rice cultivars 'Yamahoushi' and 'Murasakidaikoku'. Since 'Murasakidaikoku' is a double mutant having both dominant (purple coloration) and recessive (dwarf) markers, the somatic hybrids can be easily distinguished from their parents. Protoplasts were isolated from anther-derived calli, and electrofused protoplasts were cultured without selection of hybrid cells. Out of 27 regenerated plants, 9 proved to be hybrids based on their purple coloration and normal plant type, traits which were identical to those of the sexual $F_1$ hybrid between the same parental cultivars. The somatic hybrids included three diploid and six triploid plants. Segregation of parental markers was observed in the selfed progenies. These results demonstrated that diploid hybrids of rice could be obtained through somatic hybridization between haploid anther-derived cells instead of by sexual hybridization.

**Key words:** Anther culture – Protoplast fusion – Rice (*Oryza sativa* L.) – Somatic hybrid

**Abbreviation:** 2,4-D = 2,4-dichlorophenoxyacetic acid

## Introduction

Somatic hybridization by protoplast fusion is expected to provide a new possibility for increasing genetic variability in crops. In rice, Hayashi et al. (1987) obtained plant regeneration from somatic hybrids between cultivated rice and wild rice, and Terada et al. (1987) achieved somatic hybridization between rice and barnyard grass and the subsequent production of plantlets. In both studies, diploid cells were used as a source of rice protoplasts. Recently, we succeeded, in regenerating haploid plants from protoplasts isolated from anther-derived calli (Toriyama et al. 1986). We believe it is of great interest to know whether diploid somatic hybrids could be obtained after protoplast fusion.

In order to identify somatic hybrids, a double mutant having both dominant and recessive markers is useful (Toriyama et al. 1987b). 'Murasakidaikoku' is one such rice mutant: It shows a purple coloration over the whole plant (dominant) and is of the dwarf plant type (recessive). On the other hand 'Yamahoushi' is of the normal plant type without purple coloration. $F_1$ hybrids obtained by sexual crossing between 'Murasakidaikoku' (female) and 'Yamahoushi' show a normal plant type with purple coloration. Therefore, somatic hybrids between 'Murasakidaikoku' and 'Yamahoushi' could be expected to be distinguishable from their parents.

In this study, protoplasts were isolated from anther-derived calli of 'Murasakidaikoku' and 'Yamahoushi'. After electrofusion between their protoplasts, diploid and triploid somatic hybrids were obtained, and segregation of the two markers was compared between selfed progenies of the somatic hybrid and those of the sexual hybrid.

## Materials and methods

Anthers of rice (*Oryza sativa* L. cv 'Yamahoushi' and cv 'Murasakidaikoku') were inoculated on AA medium (Toriyama and Hinata 1985) supplemented with 0.8% agar. After 6 weeks of anther culture, the anther-derived calli were transferred to a liquid AA medium and cultured as a cell suspension. Protoplasts were isolated from a 4-month-old cell suspension in 'Yamahoushi' and from a 10-month-old suspension in 'Murasakidaikoku'. Cells were inoculated with an enzyme solution (2% Cel-

Offprint requests to K. Hinata

lulase Onozuka R-10, 0.2% Macerozyme R-10, 6.5% mannitol, and 0.1% $CaCl_2 \cdot 2\,H_2O$, pH 5.5) at 25 °C for three h (Toriyama and Hinata 1985, with some modification). Protoplasts were washed 3 times with a washing solution (6.5% mannitol, 0.1% $CaCl_2 \cdot 2\,H_2O$), and then once again with a mannitol solution (6.5% mannitol) to remove ions. Equal parts of 'Yamahoushi' and 'Murasakidaikoku' protoplasts were mixed in a mannitol solution, and their density was adjusted to $4 \times 10^5$ per ml. They were kept at 4 °C before being used in electrofusion.

Electrofusion was carried out using a Shimadzu Somatic Hybridizer (Shimadzu, Kyoto, Japan). The chamber volume is 0.8 ml, and the inter-electrode distance, 0.2 cm. Conditions of the electric fields were 2 MHz, 100 v/cm, 10 s in alternating current for pearl-chain formation and 1 kv/cm, 50 μs in direct current for fusion. After undergoing electrofusion, the protoplasts were transferred to a test tube and collected by centrifugation (100 *g*, 3 min).

Protoplast culture was carried out according to the methods of Toriyama and Hinata (1985) and Toriyama et al. (1986) with some modification. The protoplasts ($4 \times 10^5$ per ml) were cultured in 3 ml B5 medium (Gamborg et al. 1968) supplemented with 2 mg/l 2,4-D and 5% glucose. After 2 weeks of culture, 1 ml $NO_3$ medium (B5 medium without ammonium sulphate) supplemented with 2 mg/l 2,4-D and 3% glucose was added to the B5 medium, and at the beginning of the 3rd week, 1 ml $NO_3$ medium lacking glucose was added. One month after the fusion treatment, the developing calli were transferred to $NO_3$ medium supplemented with 2 mg/l 2,4-D and 1% agarose (Sigma, type I), and cultured for another 2 weeks. They were then transferred to N6 medium (Chu et al. 1975) supplemented with 0.2 mg/l indole-3-acetic acid, 1 mg/l kinetin, and 1% agarose (Sigma, type I) for shoot regeneration. Protoplasts which hadn't undergone fusion treatment were also cultured as a control.

Sexual $F_1$ hybrids were also obtained by crossing 'Murasakidaikoku' (female) with 'Yamahoushi'. Plants were grown in a greenhouse, and seeds were obtained by self-pollination for genetic analysis.

For chromosome counting, root tips, fixed with 99% ethanol-glacial acetic acid (3:1, v/v) for 1 h, were treated with an enzyme solution, and then cells were expanded on a glass slide as described in Terada et al. (1987). Chromosomes were stained with 8% Giemsa in 1/15 *M* phosphate buffer for 30 min.

## Results and Discussion

During the electrofusion of protoplasts between rice cvs 'Yamahoushi' and 'Murasakidaikoku', more than 50% of the protoplasts were involved in the fusion event, and single-pair fusion was observed in 18% of the protoplasts (Fig. 1). After the electrofusion, protoplasts began to divide on the 2nd day, and by the 6th day, about 10% of the protoplasts had divided. The protoplasts were cultured without pre-selection of hybrid cells, and small calli were obtained from about 1% of them after 1 month. The somatic hybrids between 'Yamahoushi' and 'Murasakidaikoku' could not be distinguished from their parents until they had produced shoots on the regeneration medium.

When the protoplasts of 'Yamahoushi' that had not undergone electrofusion treatment were cultured, green shoots were regenerated; purple shoots were never observed. The protoplasts of 'Murasakidaikoku' did not produce any shoots in this experiment, although we had expected them to produce purple shoots (Table 1). Therefore, any purple-colored shoots were concluded to be those of the somatic hybrids.

Of the 640 calli transferred to the regeneration medium after two independent electrofusions, 17 calli produced green shoots and were therefore considered to be unfused 'Yamahoushi' (Table 1). Nine calli produced green shoots with purple coloration (Fig. 2). These nine plants, designated YM, came into flower. All the YM plants exhibited combined characteristics of both parental cultivars (Fig. 2). Their plant height, length and thickness of leaves, and shape of panicles were normal like 'Yamahoushi'; 'Murasakidaikoku' has a height one-third that of normal plants, short and thick leaves, and small round grains that attach to rachis very close to each other. On the other hand, purple coloration of the lower leaf sheath and of the apiculus of the florets was a typical feature of 'Murasakidaikoku', while Yamahoushi did not show any purple coloration. In addition, these features were identical to those of the $F_1$ hybrid obtained by a sexual cross between 'Murasakidaikoku' (female) and 'Yamahoushi' (Fig. 2). Therefore, we concluded that all the YM plants were somatic hybrids between 'Yamahoushi' and 'Murasakidaikoku'.

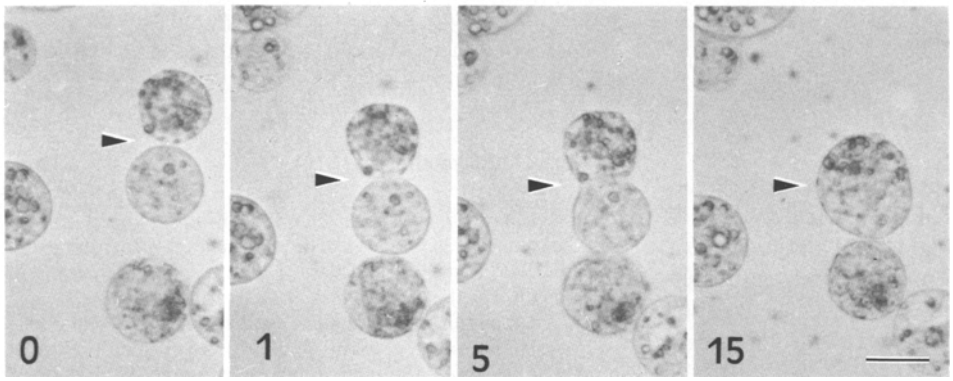


**Fig. 1.** Electrofusion of rice protoplasts (arrows). Time course of fusion was 0, 1, 5, 15 min, scale 20 μm

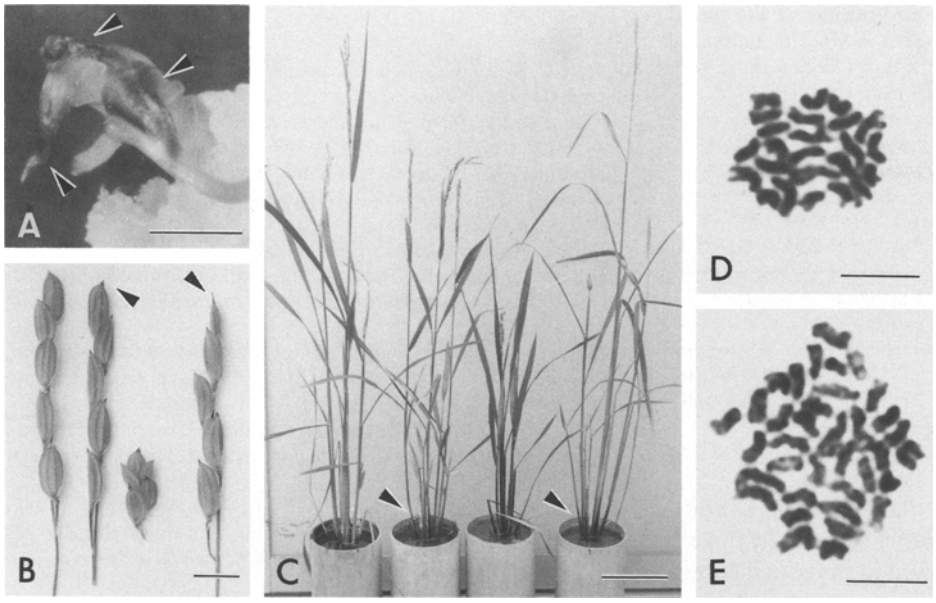


**Fig. 2.** **A** Shoot regeneration from callus after electrofusion between 'Yamahoushi' (YA) and 'Murasakidaikoku' (MD). Note the purple coloration (*arrows*). **B** and **C** (left to right) A part of the panicle (**B**) and flowering plant (**C**) of YA, the somatic hybrid (YA + MD), MD, and the sexual hybrid (MD × YA, $F_1$). **D** and **E** Chromosomes of the somatic hybrid – diploid (**D**) and triploid (**E**). Scale 5 mm (**A** and **B**), 10 cm (**C**), 5 μm (**D** and **E**)

**Table 1.** Number of calli transferred to regeneration medium (T) and number that regenerated shoots. Y: 'Yamahoushi'; M: 'Murasakidaikoku'; Y + M: electrofusion between Y and M

| | | T | Shoots | | |
|---|---|---|---|---|---|
| | | | Green | Albino | Purple |
| Exp. 1 | Y | 112 | 7 | 1 | 0 |
| | M | 112 | 0 | 0 | 0 |
| | Y + M | 320 | 12 | 1 | 5 |
| Exp. 2 | Y | 32 | 3 | 0 | 0 |
| | M | 32 | 0 | 0 | 0 |
| | Y + M | 320 | 5 | 0 | 4 |

Even though no selection for hybrid cells was made in this experiment, 9 out of the 27 regenerated plants could be shown to be hybrids (Table 1). This high frequency of somatic hybrid might be a result of a high fusion frequency induced during electrofusion, the lack of regeneration ability in one parent, and the effect of hybrid vigor on shoot regeneration. Selection is not always necessary in order to obtain somatic hybrids, as demonstrated in the *Brassica* species (Toriyama et al. 1987a).

Chromosome counts on the root tips of the YM plants showed that 3 of them had 24 chromosomes (diploid number), while the other 6 had 36 chromosomes (triploid number), (Fig. 2). In this protoplast culture system, protoplasts were isolated from an anther-derived cell suspension, and haploid (about 30%) and fertile diploid (about 40%) plants were observed in the regenerated plants (Toriyama et al. 1986; Hinata and Toriyama 1987). The cell suspension contained 30%–60% haploid cells, although the percentage varied according to the length of the subculture period and the cell lines used (unpublished data). Therefore, we are able to conclude that the diploid hybrids were produced by the fusion of two haploid protoplasts, and the triploid hybrids were produced from three haploid protoplasts or from one haploid and one diploid protoplast.

Only one diploid plant set seeds, while the other two diploid hybrids were sterile. This is the first report demonstrating seed production in somatic hybrids of graminaceous species. In the sterile plants, some anomalies could have occurred, during protoplast fusion and culture. However, further research is needed to clarify the reason for this sterility.

Segregation of the markers, plant type, and purple coloration were compared between selfed progenies of the YM plant and those of the sexual hybrid obtained from an identical cross combination. In the $F_2$ plants of the sexual hybrid, normal and dwarf plants segregated in a 3:1 ratio (Table 2), as was expected from a report that "daikoku dwarf" is controlled by a single recessive gene, *d-1* (Konoshita 1984). As for coloration at the leaf sheath of the 1-month-old seedlings of the $F_2$ plants, the segregation of purple and non-purple phenotypes was observed to be at a 27:37 ratio (Table 2), as expected from a report that anthocyanin coloration is controlled by a set of three dominant genes, *C-A-P1* (Kinoshita 1984). In

**Table 2.** Genetic analysis in the selfed progenies of the sexual hybrid (M × Y) and the somatic hybrid (Y + M). The number of seedlings with normal and dwarf plant type (A), and those with purple and non-purple leaf sheath (B) were counted

| A | Total | Normal | Dwarf | Expected ratio | $\chi^2$ | *P* |
|---|---|---|---|---|---|---|
| M × Y | 29 | 22 | 7 | 3:1 | 0.01 | 0.9–1.0 |
| Y + M | 26 | 16 | 10 | 3:1 | 2.51 | 0.1–0.2 |
| **B** | **Total** | **Purple** | **Non-purple** | **Expected ratio** | $\chi^2$ | *P* |
| M × Y | 29 | 12 | 17 | 27:37 | 0.01 | 0.9–1.0 |
| Y + M | 26 | 9 | 17 | 27:37 | 0.63 | 0.4–0.5 |

the selfed progenies of the YM plant (somatic hybrid), the segregation ratio of each marker did not fit the expected value as well as that observed in the sexual hybrid (Table 2). However, there was no significant difference (at the 5% level) in the segregation ratio between the somatic hybrid and the sexual hybrid. The results of this study clearly demonstrate that fertile diploid hybrids of rice can be obtained by somatic hybridization of haploid anther-derived cells as well as by sexual hybridization of gametic cells.

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