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Plant regeneration from cytoplasmic hybrids of rice (*Oryza sativa* L.)

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Summary. We obtained cybrid plants by electrofusing γ-irradiated protoplasts of a cytoplasmic male-sterile line "A-58 CMS" (*Oryza sativa* L.) and iodoacetamide (IOA)-treated protoplasts of the fertile (normal) rice cultivar "Fujiminori". The cybridity of the plants was confirmed by mitochondrial (mt) DNA restriction endonuclease, and plasmid-like DNA analyses, and by isozyme, cytological and morphological investigations. The chromosome number of the cybrid plants is 24.

Key words: Asymmetric protoplast fusion – Cytoplasmic male-sterility – Electrofusion – Mitochondrial DNA – Rice

Introduction

Cytoplasmic male-sterility (CMS) is a maternally inherited trait common to higher plants that causes a plant to fail to produce functional pollen grains. This CMS character is used to produce hybrid rice seeds. Transfer of the CMS cytoplasm to normal cultivars requires five to eight repeated backcrosses in order to complete the exchange of the nuclear genome of the cytoplasmic donor.

Recently, several workers have reported the successful transfer of CMS by protoplast fusion (Zelcer et al. 1978; Aviv and Galun 1980; Menczel et al. 1983, 1987). Cybrids or hybrids could be obtained by protoplast fusion within several months, whereas repeated backcrosses require several years. There has, however, been no report of the successful transmission of the CMS trait to the normal, fertile rice cultivar.

Although the list of cybrids or hybrids obtained by protoplast fusion has increased (Sidorov et al. 1981; Galun et al. 1982; Kothari et al. 1986; Endo et al. 1987; Vries et al. 1987), there are only three reports on somatic hybrids or cybrids in grass species (Ozias-Akins et al. 1987; Terada et al. 1987; Yang et al. 1988). Following fusion of protoplasts of *Pennisetum americamum* with protoplasts of *Penicum maximum,* Ozias-Akins et al. (1987) obtained three somatic hybrid cell lines, but no plants were regenerated. They indicated that the possibility of obtaining *P. maximum* (+) *P. americamum* somatic hybrid plants is currently very low. Thus, it has been impossible to efficiently recover protoplast-derived mature plants using long term suspension-cultured cell lines of any grass species. Terada et al. (1987) obtained 166 hybrid calli of rice and barnyard grass, and 44 shoots by somatic hybridization, but because of necrosis no mature plants were regenerated.

In experiments described elsewhere, ^{60}Co-irradiated protoplasts of the cytoplasmic male-sterile line "A-58 CMS" were electrofused with IOA-treated protoplasts of the fertile rice cultivar "Fujiminori". Seven cybrid and two hybrid cell lines were obtained. The hybridity of the cybrids was confirmed by analysis of their peroxidase isozymes and their mitochondrial genomes (Yang et al. 1988).

Here we report the results of our analyses of the plants regenerated from cybrid cell line 2–7. Plant morphology and peroxidase isozymes were analyzed in order to identify the nuclear genome. MtDNA restriction fragment patterns and four plasmid-like DNAs (B1, B2, B3 and B4) were used to characterize the mitochondrial genomes.

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Materials and methods

Material

Suspension-cultured cell lines of seven cybrids and two hybrids obtained as described elsewhere (Yang et al. 1988) were used.

Plant regeneration

We used two culture methods (1 and 2) to investigate the effect of different culture methods on embryoid formation and plant regeneration from cybrid colonies.

In culture method 1, 0.5 g fresh weight (FW) samples of the suspension-cultured cells were transferred to 100-ml flasks containing 25 ml of liquid N_6 basal medium, 5×10^{-6} *M* 2,4-D, 2×10^{-5} *M* ABA, 3% sucrose and 10% potato extract. The flasks were then cultured on a gyratory shaker at 100 rpm and 26 °C in the dark. After 20 day, the cells were transferred to 100-ml flasks containing 40 ml of N_6 agarose medium (0.5 mg/l kinetin, 10^{-5} *M* ABA, 3% sucrose, 15% potato extract, 1% agarose and pH 5.6). After 20 days of culture, the calli formed were transplanted onto 100-ml flasks containing 40 ml of modified N_6 regeneration medium (N_6 basal medium, $4 \times 10_M^{-6}$ BA, 3% sucrose, 15% potato extract, 1% agarose and pH 5.6), and the flasks were cultured at 26 °C in the dark.

In culture method 2, 0.5 g (FW) samples were transplanted directly to 100-ml flasks containing the modified N_6 regeneration medium and cultured as in method 1.

When the shoots of the regenerated plants had elongated to 3 cm or more, the plants were potted in autoclaved vermiculite and placed in a transparent plastic cabinet for acclimatization, after which they were transferred to a greenhouse.

Peroxidase isozyme analysis

In order to analyze the peroxidase isozymes from plants regenerated from cybrids, calli first were induced from the roots of the regenerated plants, then cell suspensions were established. Peroxidase was analyzed by isoelectric focusing as described elsewhere (Yang et al. 1988).

Characterization of the mtDNA type

For mtDNA analysis of the regenerated plants, mtDNAs were prepared from the cell suspensions established from the root-derived calli. The procedures used to isolate and digest the mtDNAs were essentially those of Yang et al. (1988). The mtDNAs were first digested with Pst I, then run on an agarose gel and stained with ethidium bromide.

Cytology

Chromosome numbers were determined by the improved method of Kurata and Omura (1981). Root tips were immersed in 2 m*M* deoxyadenosine at 30 °C for 15–20 h, then tranferred to 0.5 m*M* uridine at 30 °C for 3 h. These treated root tips were soaked in 2 m*M* 8-oxyquinoline at 20 °C for 2 h, then macerated for 50–60 min at 35 °C in a solution of 0.075 *M* HCl (pH 4.0) containing 6% pectinase (Sigma P-4625) and 6% cellulase (Onozuka R-10). After being rinsed in water, each root tip was briefly fixed (about 20–30 sec) on a slide glass with a drop of fixative (methanol : acetic acid = 3 : 1) until the fixative had thoroughly spread out. Another drop of fixative then was added, and the root tip was squashed with a needle and flame-dried. The prepared slides were immersed briefly in 100% methanol, then stained with Giemsa. More than ten nuclei were analyzed for each cybrid cell line.

Results

Plant regeneration

Two methods of embryoid formation and plant regeneration were compared. In culture method 1, 3% globular embryoids were obtained as early as 15 days after transfer of the cybrid or hybrid colonies to N_6 liquid medium, several embryoids (approximately 1%) being formed within 10 days of colony transfer to N_6 agarose medium. Approximately 0.5% of these colonies showed rapid root proliferation on N_6 agarose medium which prevented further development of the embryoids. Two germinating embryoids produced four green plants (Fig. 1 A). These plants, which continued to develop on N_6 regeneration medium, were transferred to soil for further growth (Fig. 1 B). In culture method 2, no embryoid formation was obtained and the only sign of regeneration was a few roots produced on the N_6 regeneration medium.

Morphology and chromosome number of the regenerated plants

The morphologies of all the regenerated plants were compared with those of the parents (Table 1). None of the regenerated plants was identical with sterile parent A-58 CMS; therefore, the nuclear genomes of the A-58 CMS protoplasts must have been inactivated by γ-irradiation. The morphologies of the four regenerated plants were nearly identical to the morphology of the fertile parent. Fig. 1 C and 1 E show the differences in the color of the leaf node between the two fusion parents. A-58 CMS had purple leaf nodes (Fig. 1 E), a trait encoded on the nuclear genome, whereas "Fujiminori" had colorless nodes (Fig. 1 C). The regenerated plants were the same color as the fertile parent "Fujiminori"

Table 1. Morphological characters of A-58 CMS, "Fujiminori" and regenerated plants from cybrids

	Awn	Apiculus color	Stigma color	Empty glume color	Leaf stem node color	Internode color
A-58 CMS	Awn	Purple	Purple	Purple	Purple	Purple
Fujiminori	Awnless	Green	White	Green	Green	Green
Regenerated plants	Awnless	Green	White	Green	Green	Green

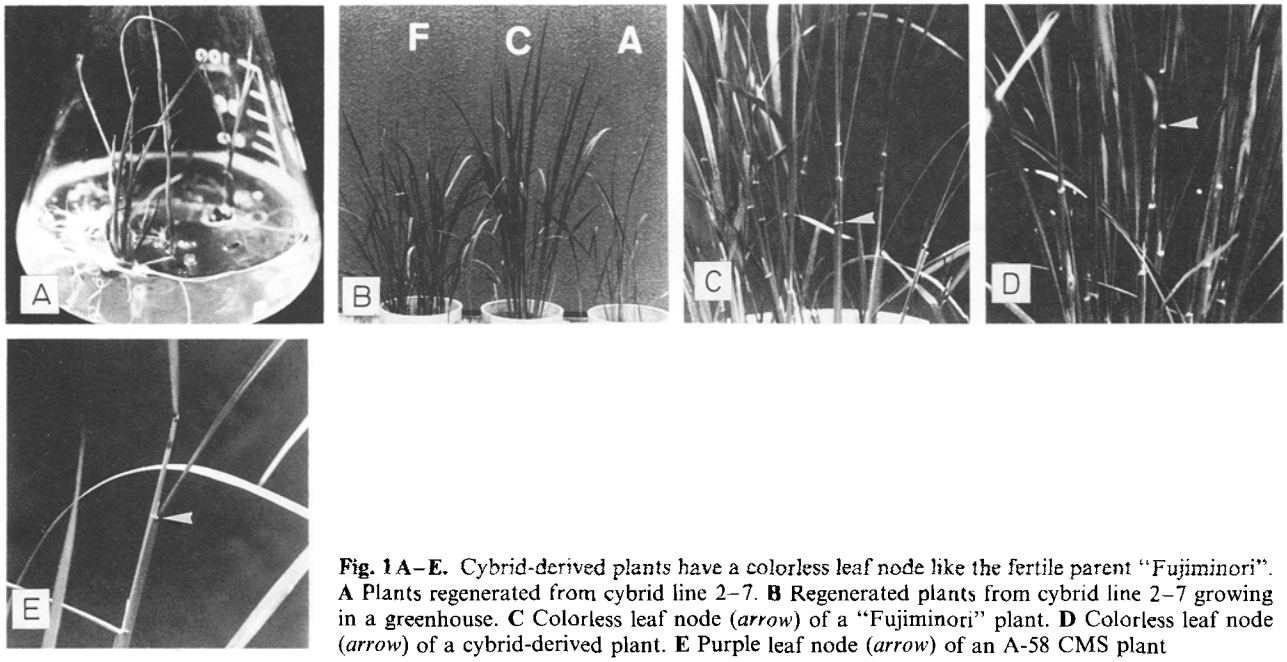

Fig. 1 A–E. Cybrid-derived plants have a colorless leaf node like the fertile parent "Fujiminori". **A** Plants regenerated from cybrid line 2–7. **B** Regenerated plants from cybrid line 2–7 growing in a greenhouse. **C** Colorless leaf node (*arrow*) of a "Fujiminori" plant. **D** Colorless leaf node (*arrow*) of a cybrid-derived plant. **E** Purple leaf node (*arrow*) of an A-58 CMS plant

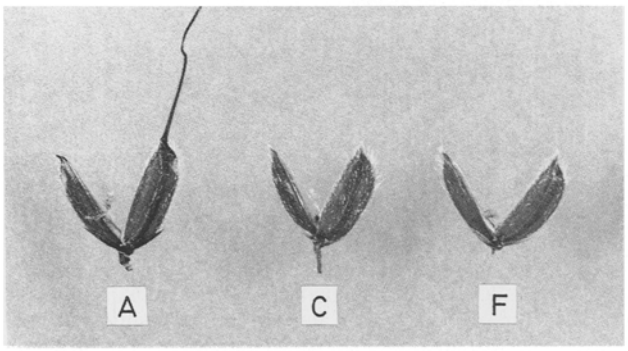

Fig. 2. Spikelet morphology of regenerated plants. **A** A-58 CMS; **F** "Fujiminori"; **C** Cybrid plant

(Fig. 1 D). Regenerated plants whose spikelet morphologies were similar to the morphology of the fertile parent "Fujiminori" are shown in Fig. 2.

The chromosome number ($2n$) for each of the four regenerated plants was 24 (Fig. 3).

Isozyme analysis

The nuclear genomes were also characterized by the peroxidase isozyme patterns of the regenerated plants. The regenerated plants showed the peroxidase isozyme pattern of Fujiminori (Fig. 4).

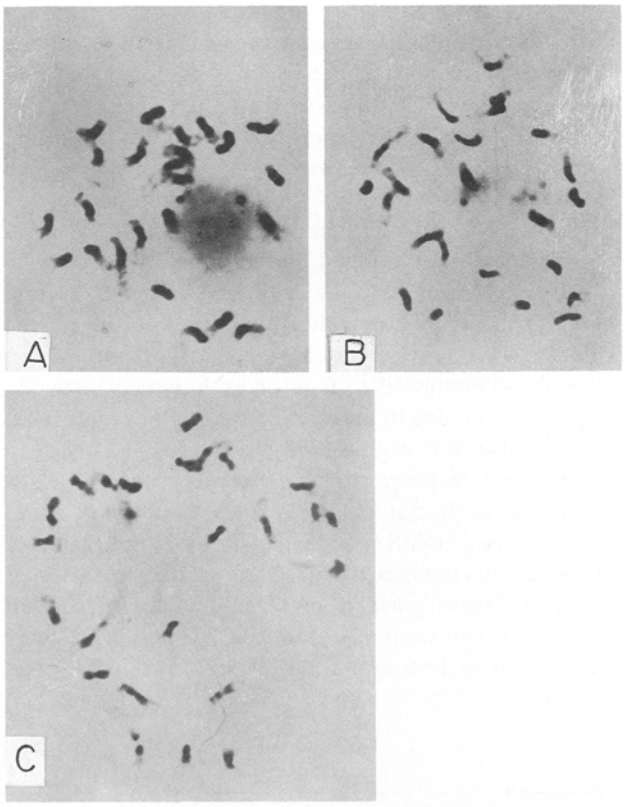

Fig. 3 A–C. Chromosome analysis of plants regenerated from cybrid cell line 2–7. **A** Plant 2-7-1 with 24 chromosomes. **B** Plant 2-7-2 with 24 chromosomes. **C** Plant 2-7-3 with 24 chromosomes

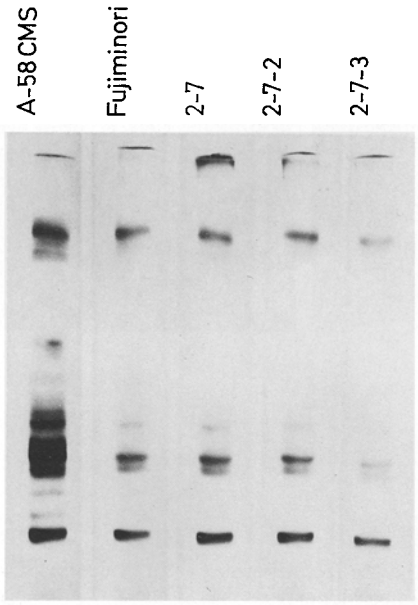

Fig. 4. Peroxidase isozyme patterns for A-58 CMS, "Fujiminori", two regenerated plants (2-7-2 and -3), and the original 2-7 cybrid cell line. The regenerated plants and their original cybrid line have patterns identical to the pattern of "Fujiminori"

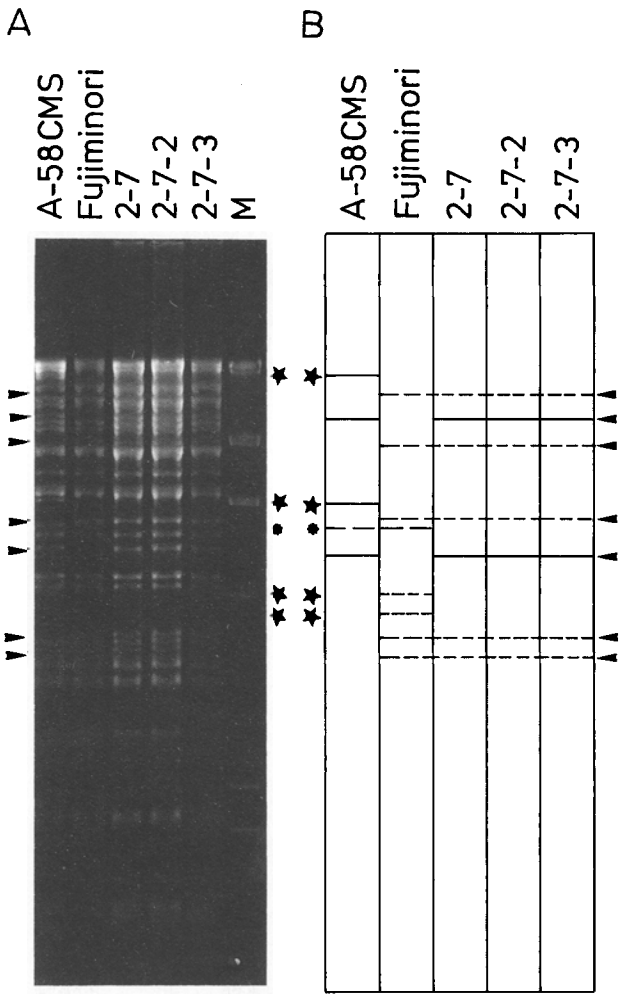

Fig. 5. **A** (left) Photograph and **B** diagram of an agarose gel after electrophoresis of mtDNAs that had been digested with Pst I. The diagram shows the unique Pst I parental bands and their distribution in regenerated plants and their original cybrid cell line (2–7). *Arrows* show the unique parental bands present in all the regenerated plants and the original cybrid cell line. *Stars* indicate unique parental bands that are absent. The *asterisks* mark the a band common to both parents that is missing in all the regenerated plants and the original cybrid cell line. For electrophoresis, a 1 μg sample of mtDNA was loaded in each well and done on an 0.8% agarose gel at 60 V for 20 h. M shows the pattern of Hind III digest of bacteriophage lambda DNA

Characterization of mtDNA

We analyzed the mtDNA of plants regenerated from the cybrids. After Pst I digestion of the mtDNAs of the regenerated plants, the restriction fragment patterns were compared. The patterns of the regenerated plants clearly differed from those of both parents, but were identical to those of the original cybrid line (2–7). For example, the parent A-58 CMS has four unique restriction fragments and "Fujiminori" has more than eight. The regenerated plants and their original cybrid cell line (2–7) had two of the four A-58 CMS Pst I restriction bands and six of the eight "Fujiminori" Pst I bands (Fig. 5, data for regenerated plants 2–7–1 and –4 not shown). A common Pst I band in both parents (asterisk, Fig. 5) was missing in the mtDNAs of all the regenerated plants and in the original cybrid cell line (2–7).

We previously reported the presence of four plasmid-like DNAs in the mitochondria of the cytoplasmic male-sterile line A-58 CMS (Shikanai et al. 1987). We therefore analyzed the undigested mtDNAs of the regenerated plants for these plasmid-like DNAs. The regenerated plants and their original cybrid cell line had four bands attributable to the four plasmid-like DNAs of A-58 CMS (Fig. 6).

Discussion

Rice plants were successfully regenerated from cybrid cells obtained by asymmetric fusion of A-58 CMS and "Fujiminori". Ours is the first report of cybrid rice plants in which the original cytoplasmic components have been partially exchanged for the γ-irradiated A-58 CMS cytoplasm. The appearance of "Fujiminori" plants with heteroplasmic cytoplasm is evidence that asymmetric protoplast fusion is effective for mitochondria transfer.

The hybrid nature of these plants has been confirmed by morphological, cytogenetic and biochemical analyses (Table 2). Many morphological traits of both the young (Fig. 1 A) and mature (Fig. 1 B) plants were similar to those of the "Fujiminori" parent (i.e. awn, color of leaf

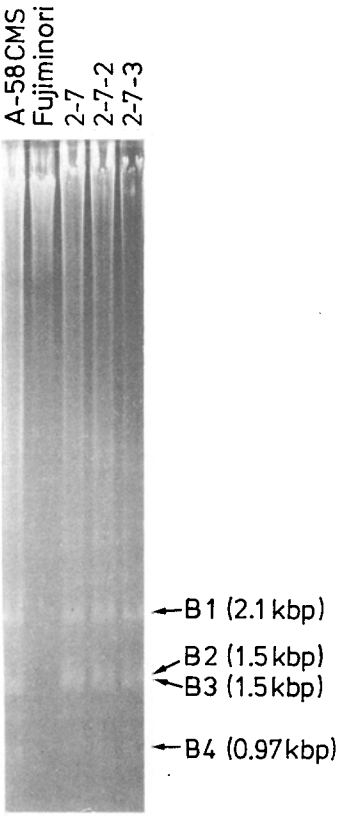

Fig. 6. Agarose gel electrophoresis patterns of undigested mtDNAs from A-58 CMS, "Fujiminori", two regenerated plants (2-7-2 and -3), and their original 2–7 cybrid cell line. Both the regenerated plants and the original cybrid cell line have the four bands attributable to the plasmid-like DNAs that originated form A-58 CMS. No such bands are present in "Fujiminori"

and stem node, and apiculus, etc., all of which are encoded on nuclear genomes). Plants also showed the "Fujiminori"-type zymogram of peroxidase. Chromosome counts showed that the cybrid plants were normal diploid number (24). We concluded that these plants had only the nuclear genome of "Fujiminori". These cybrid plants also had the mtDNA restriction patterns of the heteroplasmic type and plasmid-like DNAs of the A-58 CMS mitochondria. The appearance of cybrid plants supports our previous suggestion (Yang et al. 1988) that γ-irradiation of cytoplasmic male-sterile rice protoplasts increases the frequency of the transfer of the cytoplasmic trait alone, thereby favoring rice cybrid formation.

The mtDNA restriction patterns of the plants were distinct from those of the parents, but were identical to the pattern of the original cybrid line (2–7). This indicates that the heterogeneous populations of the mitochondria of the cybrids are very stable.

Plant regeneration was affected by the culture method used. Reports (Heyser et al. 1983; Abdullah et al. 1986) suggest that the production of satisfactorily developed embryoids is the key to obtaining a high frequency of plant regeneration from rice calli or protoplasts. Our culture method 1 proved suitable for embryoid formation and development of cybrid colonies from long-term suspension-cultured cell lines of A-58 CMS and "Fujiminori" (established more than 3 years prior to our study). One or more passages on medium containing ABA and potato extract proved effective for embryoid formation (data not shown).

Table 2. Properties of the plants regenerated from cybrids

	A-58 CMS	Fuji-minori	Cybrid plant
Marker for nuclear genome			
Peroxidase	A	F	F
Pigmentation of			
Awn	A	F	F
Apiculus	A	F	F
Stigma	A	F	F
Empty glume	A	F	F
Leaf and stem	A	F	F
Internode	A	F	F
Marker for cytoplasmic genome			
Restriction pattern of mtDNA	A	F	A+F
Plasmid-like DNA	+	−	+

A, A-58 CMS type; F, "Fujiminori" type; A+F, composed of A-58 CMS and "Fujiminori"; +, detected; −, not detected

The developmental stage of the embryoid is another important factor for plant regeneration. In culture method 1, protoplast-derived colonies were cultured on hormone-free N_6 liquid and agarose medium for 40 days to ensure sustained embryoid development. This is seen in the higher frequency of plant regeneration than was obtained by culture method 2.

In culture method 2, no embryoids or plants were obtained from colonies plated directly on hormone-free N_6 regeneration medium. Possibly no embryoid cells could develop from the very small number of embryogenic cells present in visually non-embryogenic callus. Similar results have been found for cultures of other rice protoplasts; globular and scutellar stage embryoids formed a typical leafy structure if transferred too early to light and did not develop any further (Abdullah et al. 1986).

Only a small number of globular embryoids (3%) and a few embryoids (approximately 1%) appeared on non-embryogenic callus during culture on hormone-free N_6 liquid and N_6 agarose medium (culture method 1). Smith and Street (1974) considered the decrease in the embryogenic potential of carrot callus over time (loss of embryogenic cells) to be caused by the selective advantage of

non-embryogenic cells over embryogenic cells in a mixed culture of both cell types. Our data support this hypothesis but do not exclude the possibility that the two cell types are interconvertible.

Several methods for culturing rice protoplasts have been developed (Fujimura et al. 1985; Yamada et al. 1985; 1986; Coulibaly and Demarly 1986; Toriyama et al. 1986; Abdullah et al. 1986; Kyozuka et al. 1987). However, it is difficult to eliminate all undigested single cells present in a protoplast preparation, and plantlets might be regenerated from these undigested cells. In our study, plants were obtained from fused protoplasts, which excludes the possibility of regeneration having been from undigested cells.

Our cybrid plants are now flowering, and a detailed examination (e.g., the cytoplasmic male sterility of these plants) is being made.

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