Mol Gen Genet (1988) 214:6–10

MGG

Hybrids of rice (*Oryza sativa* L.) and wild *Oryza* species obtained by cell fusion

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Summary. Cell fusion was used to obtain hybrid plants of rice (*Oryza sativa* L.) and four wild *Oryza* species, *O. officinalis*, *O. eichingeri*, *O. brachyantha*, and *O. perrieri*, to incorporate useful traits of the latter species into rice. A total of 250 mature hybrid plants were obtained by electrofusion and nurse culture methods. The hybrid nature of these plants was confirmed by karyotypic, morphological, and isozyme analysis. The hybrids of *O. sativa*+*O. eichingeri*, *O. sativa*+*O. officinalis* and *O. sativa*+*O. perrieri* produced viable pollen and the *O. sativa*+*O. eichingeri* hybrid has produced a progeny plant. Our study demonstrates the use of cell fusion for future rice improvement.

Key words: *Oryza sativa* – Gramineae – Cell fusion – Wild *Oryza* species

Introduction

Cell fusion permits hybridization of sexually incompatible species of higher plants and the transfer of cytoplasmic genomes between such species (Schieder and Vasil 1980; Schieder 1982). Thus cell fusion has been used for crop improvement in various species, particularly in the Solanaceae and the Cruciferae (Pelletier et al. 1983; Cocking 1985; Kumashiro and Kubo 1986). However, hybrid cereals, a particularly important group of plants, have not yet been obtained by this technique due to the lack of procedures available for efficient plant regeneration from protoplasts. To date only a few hybrid cell lines of the Gramineae have been produced by cell fusion (Tabaeizadeh et al. 1986; Ozias-Akins et al. 1986). Recent progress in regenerating plants from protoplasts of rice (*Oryza sativa* L.) (Fujimura et al. 1985; Yamada et al. 1986; Toriyama et al. 1986; Abdullah et al. 1986; Kyozuka et al. 1987) has made possible the application of this technique to cereals. Recently, plantlets were regenerated from somatic hybrids of rice and barnyard grass (*Echinochloa oryzicola* Vasing), which is a close relative of barnyard millet (Terada et al. 1987). However, mature plants were not obtained, presumably due to the genetic distance between these members of different subfamilies of the Gramineae (Tateoka 1957).

The genus *Oryza* consists of seven sections (Morishima 1984), some of which possess agronomically useful traits such as disease resistance, insect tolerance, and salt tolerance (Swaminathan 1986). However, the use of wild *Oryza* species for rice improvement has been hampered by the sexual incompatibility or sterility of hybrids obtained by conventional methods (Morinaga 1940; Nezu et al. 1960). Therefore, we attempted to use cell fusion as a method to incorporate useful traits of wild *Oryza* species into cultivated rice. In a series of experiments, 250 somatic hybrid plants between rice and four different wild *Oryza* species were obtained. Some of these hybrid plants have flowered and one plant has produced a progeny plant.

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Materials and methods

Plant materials. Seeds of *Oryza officinalis*, *O. eichingeri*, *O. brachyantha* and *O. perrieri* were kindly provided by Dr. Y. Sano at the National Institute of Genetics, Mishima, Japan. *O. officinalis* is resistant to insects such as brown plant hopper, green leaf hopper and white-backed plant hopper (Swaminathan 1986). *O. eichingeri* and *O. brachyantha* are resistant to bacterial leaf blight and bacterial leaf streak, respectively. All four species have extruding stigmas, which is a trait that is important for cross pollination. Three *japonica* rice varieties, Nipponbare, Aoisora and Tsukinohikari, were used as fusion partners.

Protoplast isolation, fusion, culture and plant regeneration. Calluses of wild *Oryza* species developed from germinating seeds on Murashige-Skoog agar medium with 2 mg/l 2,4-D were transferred to R2 (Ohira et al. 1973) liquid medium and 4–6 weeks later protoplasts were isolated by a procedure previously reported for rice (Kyozuka et al. 1987). Conditions for inactivation of rice protoplasts, electrofusion, culture, and plant regeneration were as described previously (Terada et al. 1987).

Isozyme analysis. A small sample (ca. 10 mg) of leaf material was used and starch gel electrophoresis and staining of enzyme activities were performed according to a published procedure (Vallejos 1983).

Chromosome analysis. Chromosomes in root tip cells of hybrid plants were examined according to the method previously described (Terada et al. 1987).

Results

Cell fusion and selection of hybrids

Rice protoplasts were inactivated by iodoacetamide prior to cell fusion to permit selection of hybrids (Terada et al.

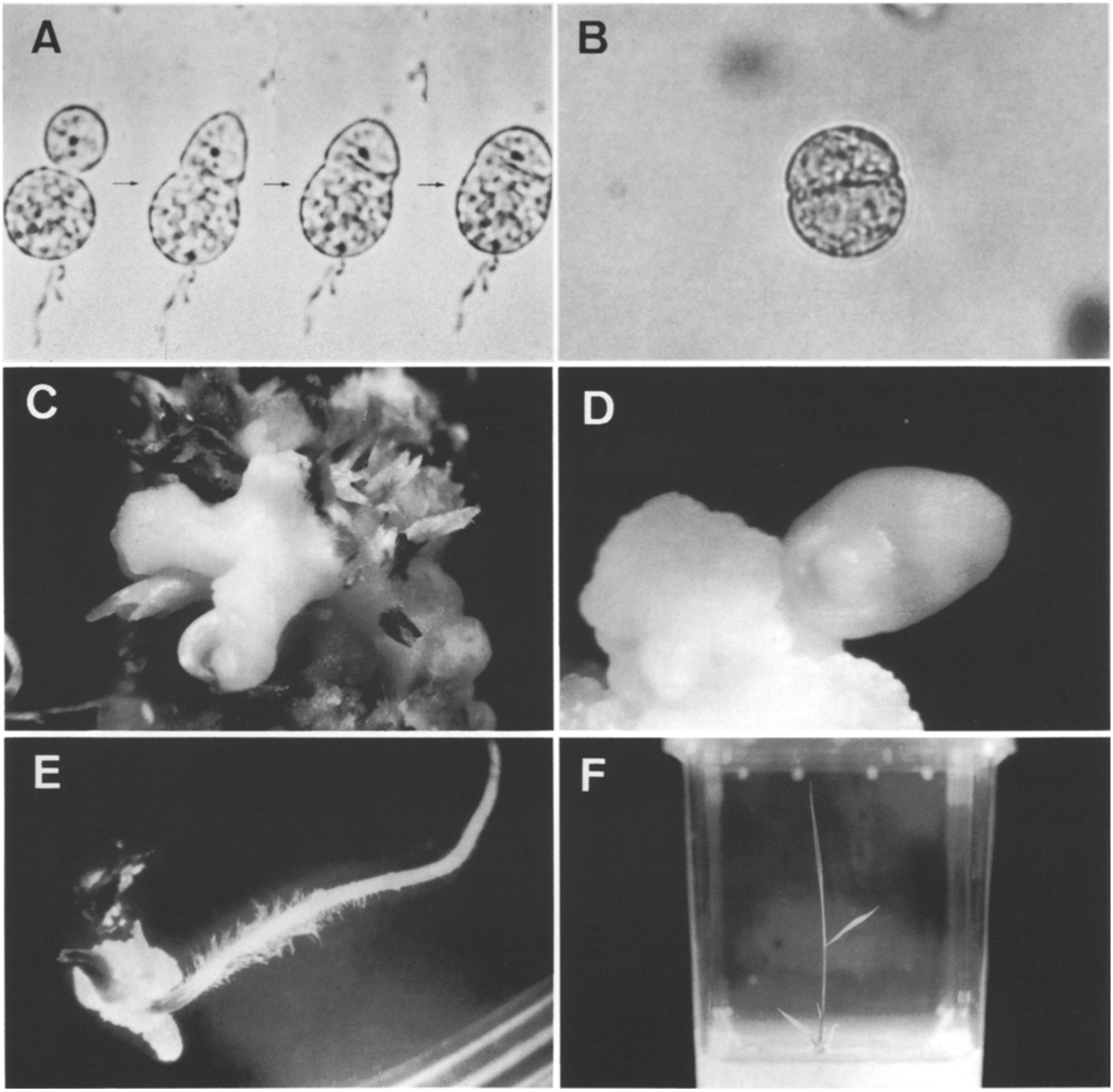

Fig. 1 A–F. Fusion, culture and plant regeneration from somatic hybrids of rice and wild *Oryza* species. **A** Electrofusion of rice protoplasts. **B** Dividing cell. **C** Morphogenic callus of rice + *O. perrieri* hybrid. **D** Somatic embryo developing from a callus of rice + *O. perrieri* hybrid. **E** Germinating somatic embryo of rice + *O. perrieri* hybrid. **F** Regenerated plantlet of rice + *O. perrieri* hybrid

1987). Because protoplasts of the wild *Oryza* species used in this study do not divide in culture, only hybrid cells were capable of division and colony formation. Electrofusion was chosen as the method of cell fusion because polyethylene glycol-based methods were not effective. The electrofusion process (3.5 kV/cm, 10 μs × 3) reproducibly produced hybrid cells in a number of experiments (Fig. 1 A, B). To eliminate rice calluses originating from protoplasts that might have escaped iodoacetamide inactivation, all calluses were screened for leucine aminopeptidase (*O. officinalis*) or esterase (*O. eichingeri*, *O. brachyantha* and *O. perrieri*) isozyme banding patterns (results not shown). On the basis of this isozyme analysis, a total of 163 calluses of four different fusion combinations were identified as hybrids (Table 1).

Morphogenesis of hybrid calli

The degree of morphogenesis (Fig. 1 C) from the hybrid calluses was variable (20% for *O. officinalis* hybrids to 61% for *O. perrieri* hybrids; Table 1). Shoots and roots were formed through somatic embryogenesis (Fig. 1 D, E) as in protoplast-derived callus of rice (Kyozuka et al. 1987). Red pigmentation was often observed on embryogenic hybrid

Table 1. Summary of plant regeneration from the hybrid callus between rice and wild *Oryza* species

Fusion combination	Number of hybrid calli	Number of hybrid calli with shoots	Number of hybrid plants
Oryza officinalis + *O. sativa*	20	4	72
O. eichinger + *O. sativa*	17	4	1
O. brachyantha + *O. sativa*	69	20	16
O. perrieri + *O. sativa*	57	35	161

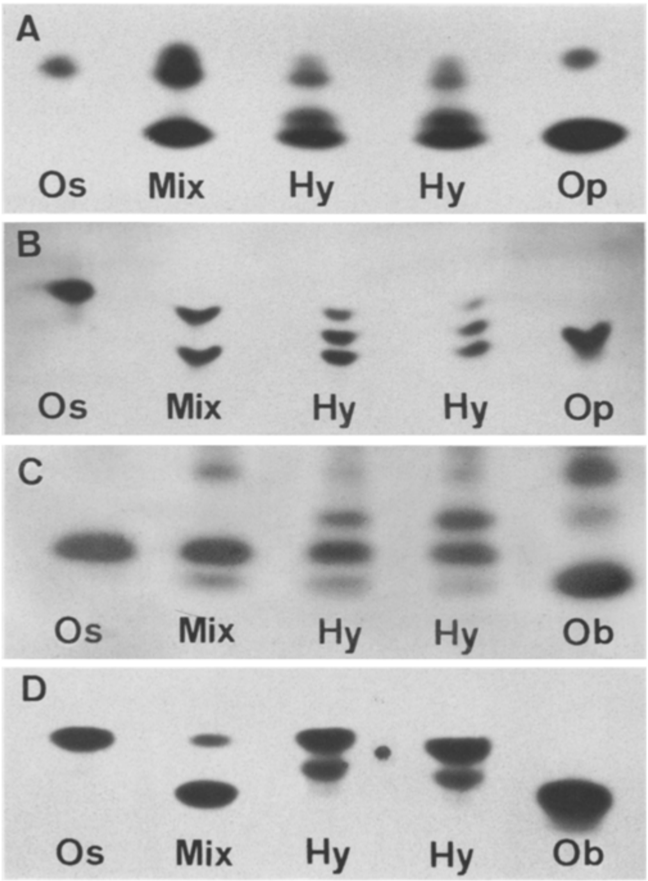

Fig. 2A–D. Isozyme analysis of somatic hybrids between rice and wild *Oryza* species. **A** Esterase pattern of rice (*Os*), *O. perrieri (Op)*, mixed control extract (*Mix*) and hybrid (*Hy*) plant. **B** Alcohol dehydrogenase pattern of rice, *O. perrieri*, mixed control extract and hybrid plant. **C** Esterase pattern of rice, *O. brachyantha* (*Ob*), mixed control extract, and hybrid plant. **D** Alcohol dehydrogenase pattern of rice, *O. brachyantha*, mixed control extract and hybrid plant

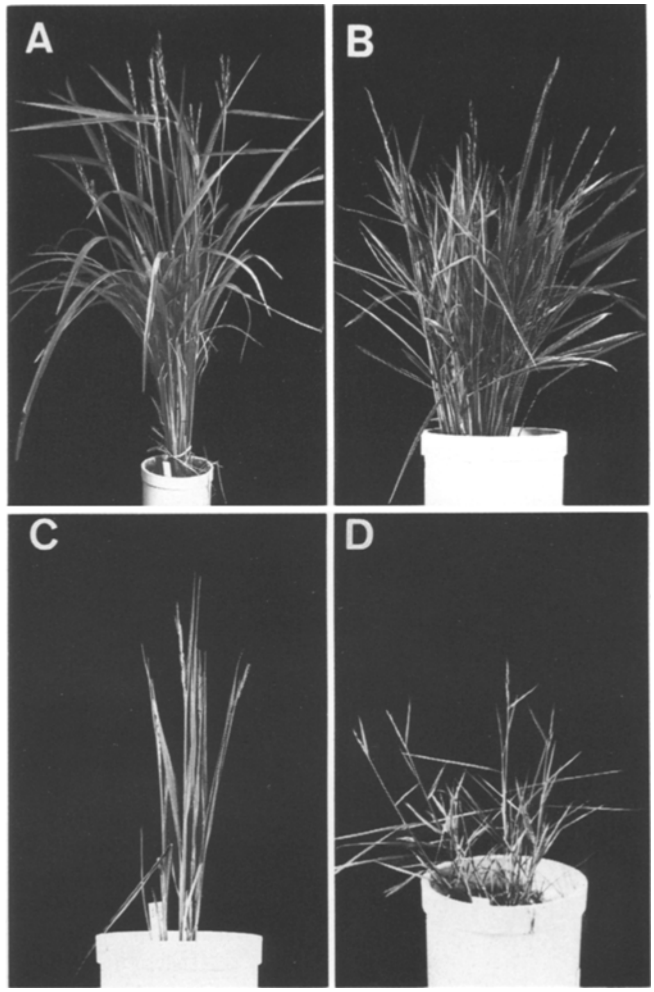

Fig. 3A–D. Morphology of hybrids between rice and four wild *Oryza* species. **A** Rice + *O. officinalis*. **B** Rice + *O. eichingeri*. **C** Rice + *O. brachyantha*. **D** Rice + *O. perrieri*

calluses but not on rice callus. A total of 250 hybrid plants were obtained, all of which grew to maturity after transfer to soil.

Analysis of hybrids

1. Isozymes. Leaf isozymes were analyzed to obtain evidence of hybridity. In the hybrids of rice + *O. brachyantha* and rice + *O. perrieri*, the presence of unique hybrid alcohol dehydrogenase (Fig. 2B, D) and esterase (Fig. 2A, C) bands not found in the mixed control extract indicated that plants were indeed hybrids and not chimeras. The hybrids of the two other fusion combinations showed similar isozyme patterns (data not shown).

2. Morphology. Morphology of the hybrids (Fig. 3) was variable depending on the species combined. The hybrids of rice + *O. officinalis* exhibited heterosis and possessed thick stems and wide leaves. The rice + *O. eichingeri* hybrid had an increased number of tillers. The hybrids of the other two combinations had phenotypes that were more or less intermediate between the parents. For example, *O. perrieri* and *O. brachyantha* are of the spreading type and this character was partially expressed by the hybrids. All the hybrids except the rice + *O. brachyantha* hybrid had extruded stigmas typical of the wild *Oryza* species. Flower development of the rice + *O. brachyantha* hybrid was abnormal. Leaves of all hybrids were a dark green color that was distinct from the leaf color of both the cultivated and the wild *Oryza* species.

3. Chromosomes. Chromosomes of root tip cells of the hybrids were counted (Table 2). Because all four of the wild

Table 2. Chromosome number of the somatic hybrid plants between rice and wild *Oryza* species

Fusion combination	Plant number	Chromosome number
O. officinalis	F-103-1	47, 48, 76, 77, 92
+ *O. sativa*	F-121-1	48, 55, 56, 56, 58
	F-130-18	53, 55, 66, 66, 68
	F-163-1	64, 74, 74, 75, 84
O. eichingeri + *O. sativa*	E-42-1	48, 48, 48, 48, 48
O. brachyantha	B-3-1	68, 70, 72, 72, 73
+ *O. sativa*	B-33-1	69, 70, 70, 72, 72
	B-41-1	65, 66, 66, 66, 67
	B-50-1	68, 69, 69, 69, 70
	B-61-1	64, 65, 65, 65, 66
O. perrieri	P-11-4	32, 36, 40, 44, 52
+ *O. sativa*	P-15-2	43, 44, 44, 47, 48
	P-22-3	36, 38, 44, 44, 48
	P-55-2	33, 44, 46, 47, 47
	P-203-7	44, 44, 46, 46, 48

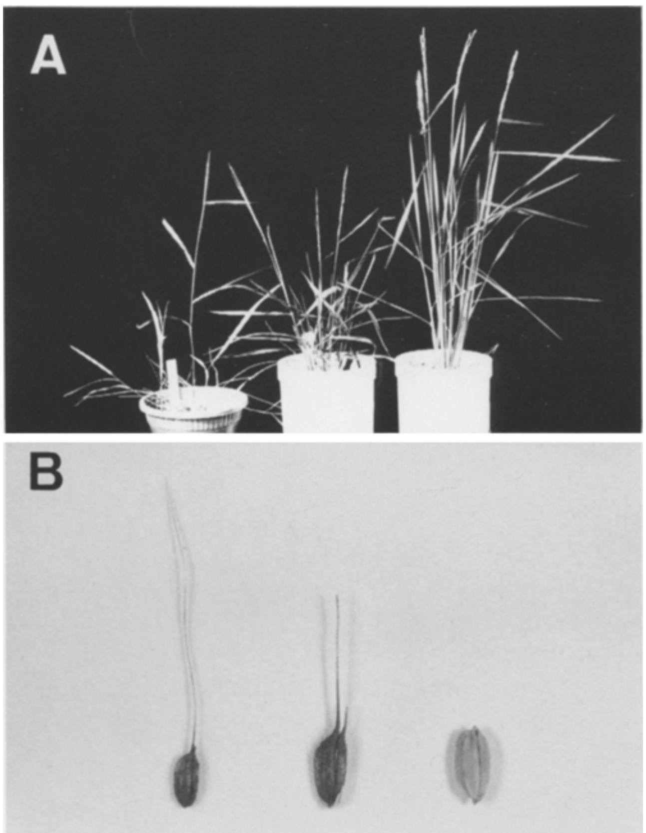

Fig. 4A–B. Morphology of the progeny of rice + *O. eichingeri* and rice + *O. officinalis* hybrids. **A** Morphology of the progeny plant (middle) of rice (right) + *O. eichingeri* (left) hybrid. **B** Morphology of the seed (middle) obtained from rice (right) + *O. officinalis* (left) hybrid

Oryza species used in this study have 24 chromosomes, the hybrids were expected to have 48 chromosomes. However, only the rice + *O. eichingeri* hybrid had the complete amphidiploid complement of 48. The three other fusion combinations produced hybrids with mixtures of root tip cells of variable chromosome number. Cells with 48(24×2) and 72(24×3) chromosomes were observed in plants of each hybrid genotype. The rice + *O. brachyantha* hybrids had relatively more consistent chromosome numbers than the two other combinations. It is not known whether this variation in chromosome number originated in culture before protoplast isolation or after cell fusion.

4. Fertility of hybrids. Hybrid plants of three different fusion combinations (rice + *O. officinalis*, rice + *O. eichingeri*, and rice + *O. perrieri*) developed normal-looking flowers. Hybrids of the combinations rice + *O. eichingeri* and rice + *O. perrieri* have produced pollen, the viability of which was determined by staining with Cotton Blue. Pollen viability was ca. 60% for one rice + *O. eichingeri* hybrid and ca. 5% for one rice + *O. perrieri* hybrid. These plants set seeds that increased in size normally. However, normal embryo and endosperm development was observed only for the former hybrid. One of the progeny of the rice + *O. eichingeri* hybrid grew to maturity (Fig. 4A). The rice + *O. officinalis* hybrid, whose pollen viability was not examined, set one seed with the intermediate phenotype (Fig. 4B). Backcrosses with rice and embryo culture are currently being attempted. Rice + *O. brachyantha* hybrids have not yet produced pollen.

Discussion

By electrofusion of protoplasts and use of nurse culture method we have demonstrated the feasibility of routinely producing hybrids of rice and wild *Oryza* species. Hybridity of these plants was confirmed by different observations; (i) the hybrids contained unique alcohol dehydrogenase and esterase isozyme bands that were not found in either parent or mixed control, (ii) the hybrids showed the unique intermediate morphology in plants, seeds and the progeny plant, and (iii) the chromosome number of the hybrids was variable in the range of 32–92 which was markedly different from protoplast-derived rice plants whose chromosome number (24) was very stable (Terada et al. 1987). At this time, seeds have been obtained only from rice + *O. eichingeri* and rice + *O. officinalis* hybrids. Transfer of limited amounts of the genomes (Bates et al. 1987; Dudits et al. 1987) of wild *Oryza* species by X-irradiation of protoplasts prior to fusion has been tried and a number of plantlets are now developing.

The methods described in this study should be useful for incorporating into cultivated rice a variety of agronomically useful traits found in wild *Oryza* species. For example, the salt tolerance of *O. coarctata* (*Sclerophyllum coarctata*) and the drought resistance of *O. australiensis* are important traits that might be transferred to rice by this method. Cell fusion might also be used to transfer various traits encoded by cytoplasmic genomes. Several types of cytoplasmic male sterility (CMS) have been identified in rice (Shinjo 1984) and used for the production of hybrid rice. By eliminating the numerous backcrosses that are required by conventional methods, the cell fusion procedure described here would offer a far more efficient method by which to transfer CMS to other cultivars.

Acknowledgements. We thank Drs. Y. Sano and H. Morishima at the National Institute of Genetics, Mishima, Japan for supplying seeds of wild *Oryza* species, and Dr. R. Chaleff for critical reading of the manuscript. We also thank Y. Yahiro for continuous encouragement and suggestions, and S. Nishibayashi and K. Ito for help in chromosome counting.

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Communicated by J. Schell

Received April 19, 1988