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Detection of specific chromosome reduction in rice somatic hybrids with the A, B, and C genomes by multi-color genomic *in situ* hybridization

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Abstract A multi-color genomic *in situ* hybridization (McGISH) method has been developed. Three different rice genomes, A, B and C, involved in rice somatic hybrids were distinguished using three different fluorescent signals. All the rice chromosomes from the different genomes could be identified by different fluorescent colors, and the distribution of each genome in the nucleus was clearly visualized under a fluorescence microscope. The relationship between chromosomal constitution and morphological variations observed in the somatic hybrids, and the utility of McGISH, are discussed based on the results currently obtained.

Key words Genomic *in situ* hybridization (GISH) · *Oryza sativa* and *O. punctata* · Somatic hybrids · Cell fusion · Chromosome reduction · A, B and C genomes

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Introduction

Somatic hybridization is virtually the only major tool available for generating hybrids between genetically distant plant species. Several somatic hybrids between tomato and a wild egg plant (Escalante et al. 1998), rice and its wild relatives (Hayashi et al. 1988; Mori and Kinoshita 1991), tobacco and its wild relatives (Kenton et al. 1993; Kitamura et al. 1997), and *Crocus* (Ørgaard et al. 1995) have been generated successfully by somatic hybridization. Even combinations between phylogenetically quite distant species, such as rice and carrot (Kisaka et al. 1994), and barley and carrot (Kisaka et al. 1997), have been obtained successfully. It is generally difficult to obtain progeny by ordinary cross hybridization of these combinations.

The main problem of somatic hybrids and their progeny lies in genetic instability. It is quite often the case that the somatic hybrids and their progeny show complete sterility. Various chromosome aberrations have been identified as the major cause of these genetic deficiencies. Thus, research in this area has been important because it provides the means of verification and characterization of the variation occurring in somatic hybrids. Kao (1977) carefully observed chromosomal behavior in somatic hybrids between soybean and *Nicotiana glauca*, whose chromosomes could be distinguished according to their sizes. Babiychuk et al. (1992) also examined the chromosomal constitution of somatic hybrids between *Nicotiana tabacum* and *Atropa belladonna* on the basis of differences in the sizes of their chromosomes. As for somatic hybrids between *Brassica* species, Yamashita et al. (1989) verified the hybrid nature of the regenerated plants on the basis of the presence of the small satellites of *B. oleracea* and the large satellites of *B. campestris*. It is, however, difficult to examine the chromosomal behavior and constitution in the somatic hybrids when species with similar chromosomal morphology and no distinguishable

markers are used as the parents, as in hybrids between species of the same genus.

Recent advances in molecular cytology, especially in an *in situ* hybridization method, have made it possible to identify different genomes in plants. Schwarzacher et al. (1989) used total genomic DNA from one parent and succeeded in obtaining clear differential painting of the chromosomes in the hybrid between wild barley and wild rye species. They named the method genomic *in situ* hybridization (GISH). Subsequently, this method has been applied extensively to identify chromosomes belonging to different genomes, e.g., in hybrids between tobacco and its wild relatives (Kitamura et al. 1997) and in the progeny of somatic hybrids between tomato and a wild relative of egg plant (Escalante et al. 1997). Even in two amphidiploid wild rice species with BBCC and CCDD genomes, each genome could be clearly identified by GISH in conjunction with an imaging method (Fukui et al. 1997).

In this paper, we describe the development of multicolor genomic *in situ* hybridization, McGISH, using three different fluorescent colors to discriminate the three different genomes, A, B and C, involved in the somatic hybrids between cultivated rice species (AA) and wild rice species (BBCC). Furthermore, the specific chromosome reduction which occurs only in the B and C genomes was revealed by McGISH. The effectiveness of McGISH for the analysis of chromosomal aberrations is discussed in relation to the sterility and morphological variation common in the somatic hybrids and their progeny.

Materials and methods

Production of somatic hybrids by asymmetric cell fusion

Induction of seed calli, cell fusion, and plant regeneration were carried out following the methods described by Kyozuka et al. (1987). The isolated protoplasts from one of the parents, *O. sativa* (AA), were treated with 30 m*M* iodoacetamide (IOA) for 20 min at 4°C in 5% MES-KMC (equal volumes of 0.35 *M* KCl, 0.245 *M* $MgCl_2$ and 0.254 M $CaCl_2$) at pH 5.6 prior to the cell fusion. For the other parent, *O. punctata* (BBCC), we selected a line lacking the ability to regenerate plants from calli. An electrofusion apparatus (SSH-1, Shimazu) was used for the cell fusion.

Identification of somatic hybrids

Isozyme analysis

The young leaf extracts of regenerated hybrids in *in vitro* condition were used. Polyacrylamide gel electrophoresis and staining of enzyme activities for esterase, peroxidase and alcohol dehydrogenase were performed according to standard methods.

Restriction fragment length polymorphism (RFLP) analysis

For the hybrids screened by isozyme analysis, we then employed RFLP analysis to distinguish the hybrids. Total DNA was isolated from the leaves of the somatic hybrids by the CTAB method (Murray and Thompson 1980). Broad bean *Eco*RI fragments, which contain rDNA with a non-transcribed spacer region, were Southern-hybridized with total rice DNA digested with *Bam*HI, *Eco*RI, *Xba*I, *Sma*I, *Sal*I, *Hpa*I and *Kpn*I.

Chromosome preparation

Root tips, 1–2 cm long, were excised and fixed in an ethanol-acetic acid (3 : 1) fixative after pretreatment with 2 m*M* 8-hydroxyquinoline solution for 4 h at room temperature. Chromosome samples were prepared by an enzymatic maceration/air-drying (EMA) method on glass slides (Fukui 1996). Prior to GISH, they were treated with 100 mg/ml RNase A (Sigma) in 2 × SSC at 37°C for 60 min, dehydrated through a 70%, 95%, and 99% ethanol series for 10 min each, and air-dried.

Multi-color genomic in situ hybridization (McGISH)

Total DNA of diploid rice species, *O. sativa* (AA), *O. punctata* (BB), and *O. officinalis* (CC) were labeled with biotin-16-dUTP (Boehringer Mannheim) or digoxigenin-11-dUTP (Boehringer Mannheim) by the standard random primed labeling protocol. The hybridization mixture (100 ng labeled probe/slide dissolved in a mixture of equal parts of 50% formamide and 2 × SSC) was denatured for 10 min at 90°C and then immediately cooled down to 0°C. The chromosome spreads were denatured in 50% formamide/2 × SSC for 6 min at 70°C with the hybridization mixture on a modified thermal cycler (Fukui et al. 1994; PHC-3, Techne, Cambridge, UK) and then hybridized for 3–4 days at 37°C. After hybridization they were washed twice in 2 × SSC, once in 50% formamide/2 × SSC, and once in 4 × SSC, each for 10 min at 40°C. Fluorescein-avidin (1%, Vector Laboratories, Calif.) in 1% bovine serum albumin (BSA) in BT buffer (0.1 *M* sodium hydrogen carbonate, 0.05% Tween-20, pH 8.3) was dropped onto the chromosome spreads, which were then incubated at 37°C for 60 min. After the fluorescein-avidin solution was rinsed with BT buffer three times at 37°C for 10 min each, the biotinylated anti-avidin (1%, Vector Laboratories) for secondary amplification and the anti-digoxigenin-rhodamine raised in sheep (10%, Boehringer Mannheim) in 5% goat serum in BT buffer were dropped onto the chromosome spreads, and they were then incubated at 37°C for 60 min. After brief washing with BT buffer three times at 37°C for 5 min each, 1% fluorescein-avidin and anti-sheep-Texas Red (1%, Vector) in 1% BSA in BT buffer were dropped onto them, and they were incubated at 37°C for 60 min. After rinsing them twice with BT buffer, and once with 2 × SSC, at 40°C for 10 min each, the chromosome spreads were finally counterstained with 1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) in an antifadant solution (Vector Shield, Vector). Before each immunocytochemical step they were blocked with 5% bovine serum or goat serum albumin in BT buffer at 37°C for 5 min.

The chromosome spreads were observed with a fluorescence microscope (DMRXE, Leica) with a high-sensitivity cooled CCD camera (PXL 1400, Photometrics). The B- and G-light excitation filters were used for the detection of the fluorescein and rhodamine or Texas Red signals, respectively. The signal images were digitally stored in a personal computer (Quadra840AV, Macintosh) and analyzed by imaging software (IPLab Spectrum, Image and measurement, Signal Analytics).

Results

Some hundreds of somatic hybrids were generated by cell fusion between *O. sativa* cv 'Kitaake' (AA, 2n = 24)

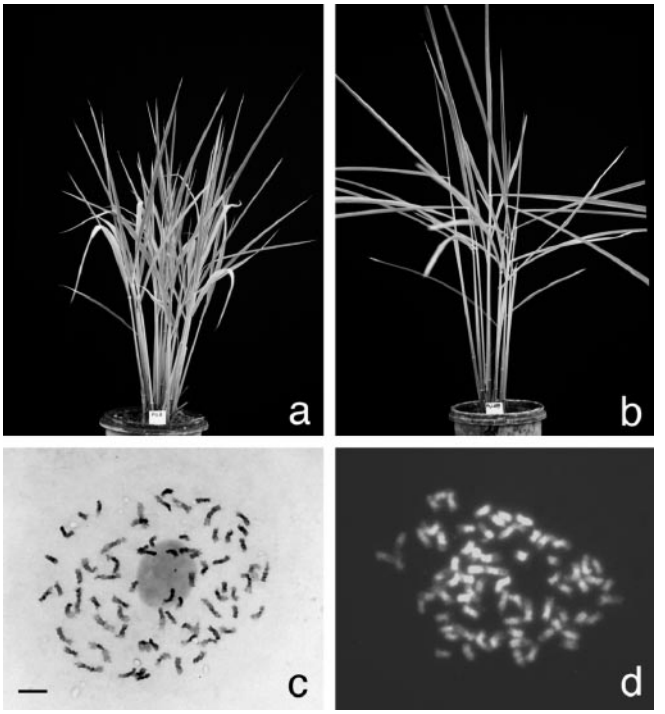


Fig. 1a–d Rice somatic hybrids and their chromosome complements. **a** and **c** PU2 and its chromosome complement stained with Giemsa solution (2n = 6x = 72); **b** and **d** PU289 and its chromosome complement stained with DAPI (2n = 6x = 72). *Bar*: 5 μm

and *O. punctata* (BBCC, 2n = 48), and the progeny were obtained by subsequent embryo rescue techniques. Two lines, PU-2 and PU-289, were selected for this study. Figure 1 shows the two rice plants and their chromosome complements. PU-2 showed the expected chromosome number of 72, while some chromosomes were often missing in PU-289 – between 65 and 72 chromosomes were counted in the roots. Both lines exhibited anomalies in the morphology of the plants, and their heights were less than those of either of their parents. Compared with PU-2, PU-289 showed a much more vigorous growth habit, especially on plant height, spikelets per panicle, and flag leaf length (Table 1). In addition, PU-2 showed delayed heading.

Although three leaf isozymes, i.e., esterase, peroxidase, and alcohol dehydrogenase, were analyzed, only esterase and peroxidase patterns showed obvious differences between the parents. Thus, esterase and peroxidase were used to confirm the hybridity. Two types of isozyme patterns were detected for both esterase and peroxidase. One is the same band pattern as *O. sativa* and the other is a unique one, different from both the parents. From these results, we could eliminate undesired non-hybrid plants which were derived from *O. sativa*. For the RFLP analysis, the digestion of total DNA with *Bam*HI exhibited the most distinct polymorphism of rDNA between the parents. PU2 showed the same band pattern as one of the parents, *O. punctata*, while PU289 showed bands from both parents.

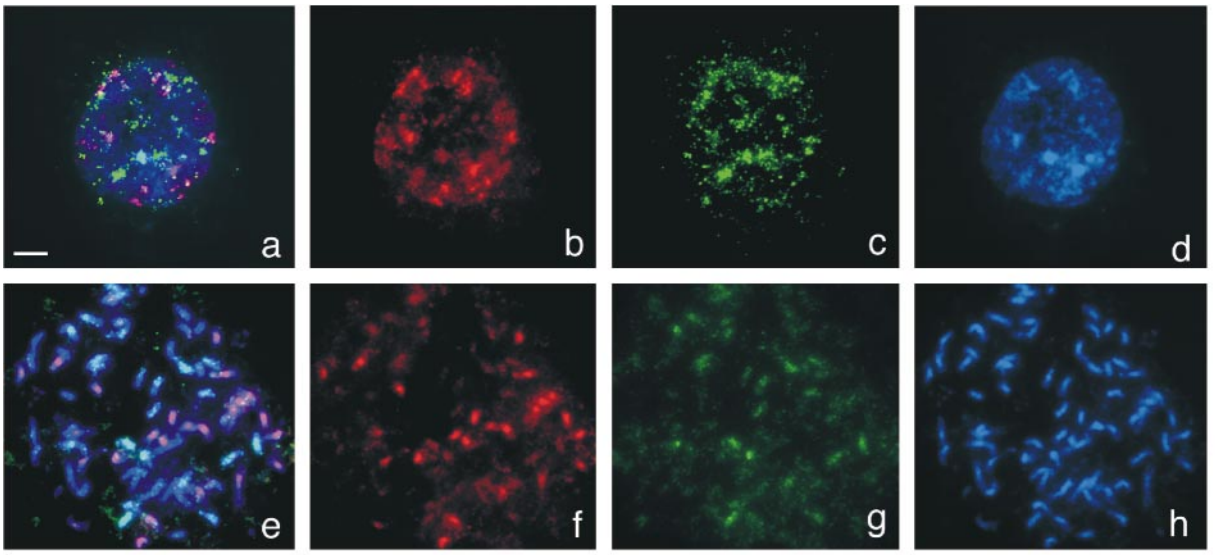
Figure 2 shows multi-color GISH of PU2 with the two genomic probes of the A (red) and C (green) genomes. Seventy-two chromosomes are visualized in most of the cells by counter-staining with DAPI (Fig. 2h). Twenty-four red chromosomes belonging to the A genome (Fig. 2f) and 24 green chromosomes belonging to the C genome (Fig. 2g) were detected individually. Finally, the 24 chromosomes derived from each of the A, B, and C genomes were completely identified (Fig. 2e). Only one case of chromosome insertion was detected. Figure 2d shows the nuclear region counterstained with DAPI, which revealed the uniform distribution of DNA in the nucleus except for the region of the nucleolus. On the other hand, the non-uniform distribution of each genome-specific DNA was detected (Fig. 2b,c). Each genome seemed to occupy its own domain around the nucleolus, which was unstained as if it were a small cavity (Fig. 2a).

Figure 3 shows McGISH of PU289 with the two genomic probes for the A (red) and B (green) genomes (upper panels) or A (red) and C (green) genomes

Table 1 The morphological and biochemical characteristics of the two lines and the parents

Lines	Plant height (cm)	Panicle length (cm)	Spikelet number per panicle	Flag leaf length (cm)	Flag leaf width (cm)	Spikelet length (cm)	Spikelet width (cm)	Isozyme band pattern[a]	rDNA band pattern[b]	Stigma color[c]	Remarked character
O. sativa (AA) cv Kitaake	53.0	21.8	28	22.5	1.2	6.7	3.5	C	C	W	
O. punctata (BBCC) (W1564, NIG)[d]	99.0	23.1	98	23.8	1.7	5.4	2.1	W	W	P	
PU-2 (AABBCC)	45.2	11.4	26	20.0	1.8	7.4	3.0	U	W	P	delayed heading
PU-289 (AABBCC)	51.0	17.0	47	47.2	2.0	8.3	2.8	U	W+C	P	

[a] C, Cultivar type; W, wild type; U, unique zymogram for esterase and peroxidase
[b] C, Cultivar type; W, wild type
[c] W, white color; P, purple color
[d] NIG: National Institute of Genetics, Japan



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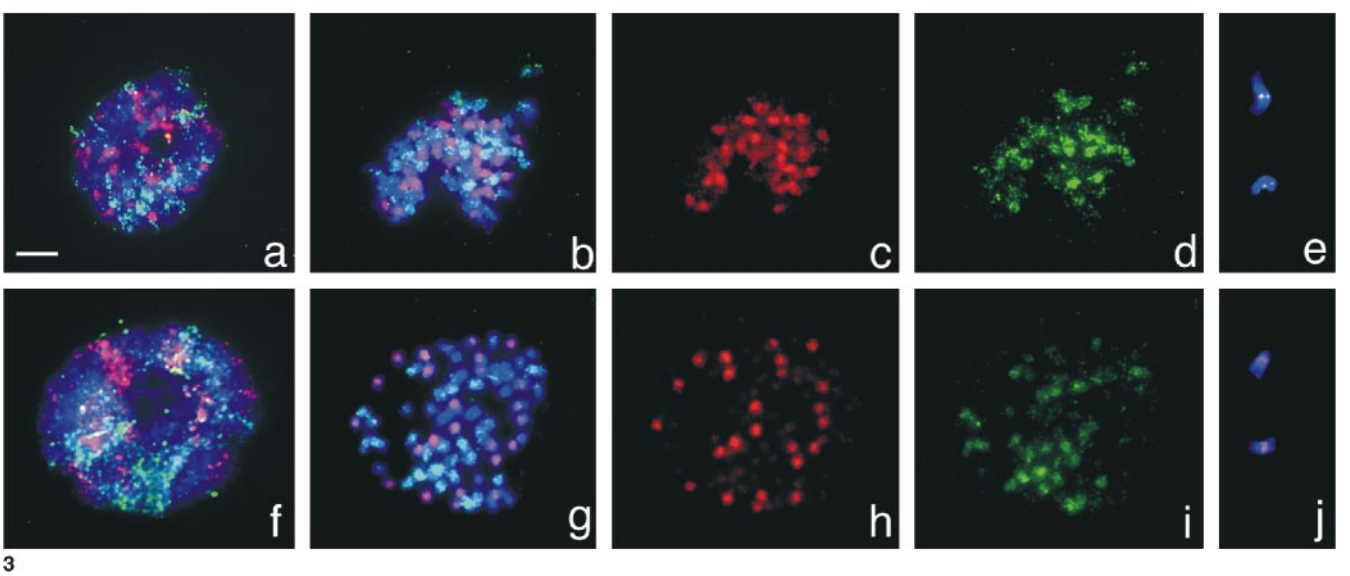

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Fig. 2a–h McGISH of PU-2 with the two genomic probes of the A (*red*) and C (*green*) genomes. Counter-staining was applied with DAPI (*blue*). **a** and **e** Discrimination of the three different genomes in a nucleus and a chromosome complement. **b** and **f** Distribution of the A genome within the nucleus and detection of the A genome chromosomes with *red fluorescence*. **c** and **g** Distribution of the C genome within the nucleus and detection of the C genome chromosomes with *green fluorescence*. **d** and **h** DAPI-stained nucleus and chromosomes. *Bar*: 5 μm

Fig. 3a–j McGISH of PU-289 with the different combination of the genomic probes, the A and B (*upper panels*) or the A and C (*lower panels*) genomes, respectively. **a** Discrimination of the three different genomes with *red* (A genome) and *green* (B genome) *fluorescence* in the nucleus. The nucleus was counter-stained with DAPI (*blue*). **b** Discrimination of the chromosomes belonging to the three different genomes with *red* (A genome) and *green* (B genome) *fluorescence*. **c** and **h** Detection of the A genome chromosomes with *red fluorescence*. **d** Detection of the B genome chromosomes with *green fluorescence*. **e** Insertion of the B genome chromosomal fragments into the C genome chromosomes detected. **f** Discrimination of the three different genomes with *red* (A genome) and *green* (C genome) fluorescence in the nucleus. **g** Discrimination of the chromosomes belonging to the three different genomes with *red* (A genome) and *green* (C genome) *fluorescence*. **i** Detection of the C genome chromosomes with *green fluorescence*. **j** Insertion of the A genome chromosomal fragments into the B genome chromosomes detected. *Bar*: 5 μm

(lower panels). Because the chromosome number of PU289 was more unstable than that of PU2, different combinations of genomic probes were employed to confirm the genomic composition. After counterstaining, 65–72 chromosomes were counted, and McGISH could identify the chromosomes belonging to each of the genomes (Fig. 3b, g). It was revealed that the chromosomes from the A genome were never eliminated from the nucleus and that the expected chromosome number of 24 was always present (Fig. 3c, h). On the other hand, a loss of B genome chromosomes was observed frequently, while the elimination of chromosomes from the C genome was detected less frequently. For example, 22 and 23 chromosomes belonging to the

B and C genomes, respectively, were detected within the total of 69 chromosomes in Fig. 3b and 3d, and 17 and 24 chromosomes belonging to the B and C genomes, respectively, were detected among the total of 65 chromosomes in Fig. 3g and 3i. Figure 3e and 3f show chromosomal aberrations, with the insertion of the B-genome chromosomal fragments into C genome chromosomes, and of A genome, chromosomal fragments into B genome chromosomes, respectively. These types of insertions and translocations were observed frequently. In the nuclei, each genome also occupied its own domain around the nucleolus as in the case of PU2 (Fig. 3a, f), which was best depicted in Fig. 3f.

The elimination of chromosomes was also observed in some cells from PU2. Even in such cells from PU2, the same tendency of chromosome elimination as in PU289 was observed; i.e., PU2 also contains all 24 chromosomes of the A genome, and elimination of the chromosomes from *O. punctata* usually occurred.

Discussion

There are many species with various genomic compositions in wild species of *Oryza*. Wild rice species have been identified as having several useful traits, such as disease resistance, insect resistance, cold weather resistance, and salt tolerance (Swaminathan 1986). Many efforts have been made to introduce the useful traits from wild species to cultivated rice, and their successful introduction has been reported (Multani et al. 1993; Ishii et al. 1994). The final objective in producing somatic hybrids between *O. sativa* and *O. punctata* using the cell fusion method is also the introduction of useful traits from the wild species.

It is well-known that variations are found in plants produced by fusion. The genomic stability may result in sterility and variation in the hybrids. Without exception, the somatic hybrids between *O. sativa* with the A genome and *O. punctata* with the B and C genomes generated by cell fusion used in this study showed both complete sterility and many morphological differences from the parents and between the two lines. For example, PU-289 grew much more vigorously than PU-2. RFLP analysis using rDNA as the probe showed that PU-2 had the same band pattern as *O. punctata* but that single bands from both parents were found in PU-289. This result indicates that the nucleus of PU-2 has lost 45S ribosomal RNA genes from *O. sativa* even though the chromosomes are completely maintained.

Diversity of chromosome number was also observed in both somatic hybrids, not only between different somatic hybrids but also within an individual plant. Hitherto it was not known how the chromosomes belonging to different genomes behaved in the somatic hybrid, and how each genome was distributed within the nucleus. The use of McGISH made it possible to answer these questions visually. The chromosome number of PU-2 was comparatively stable and usually 72. However, some chromosomes tended to be eliminated in the case of PU-289. Interestingly, such elimination occurred only for the chromosomes from *O. punctata*, especially the chromosomes from the B genome. The chromosomes derived from *O. sativa* were never missing.

These somatic hybrids were grown at a rather low temperature and in dry conditions, which are characteristic of the most northern part of Japan (Hokkaido area, latitude 41°–46°N). The sources of their parents are quite different. One parent, *O. sativa* cv 'Kitaake' is the domestic variety of Hokkaido, but the other parent, *O. punctata*, is a tetraploid species which is distributed mostly in the central part of tropical Africa. It is likely that retention of predominantly genetic factors from *O. sativa* would be reasonable in order to adapt to the conditions of the Hokkaido area. The reduction in the number of chromosomes from *O. punctata* may provide better genetic conditions for adapting to the environment and may result in the vigorous growth habit of PU289.

The McGISH method has been applied to the somatic hybrids, and is effective for identifying chromosomes and examining the distribution of each genome in the nucleus. Jacobsen et al. (1995) employed the GISH method for the identification of alien chromosomes in the backcross progeny of fusion hybrids between potato and tomato, the chromosomes of which were small and similar to each other; they could discriminate tomato chromosomes from potato chromosomes and showed the usefulness of this method for plant breeding programs. In this study, we used two genomic probes simultaneously and succeeded in discriminating the chromosomes in the three different genomes of the somatic hybrids between rice and its wild relatives with small and similar chromosomes.

The two different chromosome sets of amphidiploid wild rice were distinguished with one genomic probe (Fukui et al. 1997). This is the first report on the employment of McGISH with two probes for *Oryza* species having three kinds of chromosomes. The McGISH method shows considerable possibilities for the characterization of other somatic hybrids and should provide indispensable information on genomic organization. Furthermore, it is expected that results obtained by McGISH will help us to understand the general process of genome evolution in both somatic and sexual hybridization.

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