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Physical Mapping of Rice DNAs by an Improved FISH Method

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Abstract
An improved fluorescence *in situ* hybridization (FISH) method for localizing genes and DNA sequences on rice chromosomes has been developed in our laboratory. Three improvements in the fluorescence *in situ* hybridization procedures, i.e. development of four steps of post-treatments, development of a modified thermal cycler and introduction of an imaging method enabled to obtain clear and reproducible signals on rice chromosomes. The improved FISH method was employed for physical mapping of 45S ribosomal RNA genes in *Oryza* species. Variability in the number of 45S rDNA loci was detected among eight diploid species. The FISH method was also applied to localize the rice repeated sequences. The repeated sequences were adjacent to the telomeric regions of the long arms of chromosomes. Furthermore, a multi-color FISH (McFISH) method was developed and was applied to characterize *O. glaberrima* chromosomes.

Discipline: Biotechnology/Experimental apparatus and methods
Additional key words: 45S ribosomal RNA gene (45S rDNA), tandem repeated sequences, multicolor FISH (McFISH)

Introduction

In situ hybridization (ISH) was first reported in the late 1960s as a cytogenetical method to visually detect specific DNA or RNA sequences located on the chromosome, nucleus, cell, or tissue samples using a microscope[11]. A non-RI ISH method was developed using biotin labeling in the early 1980s[17]. The new ISH method, especially the fluorescence *in situ* hybridization (FISH) method enabled to attain a high sensitivity, shorten the detection time, and facilitate the handling of samples for gene mapping.

In plant there are several reports on the detection and mapping of genes using the FISH method, i.e. wheat[19], barley[18], rye[1] and tobacco[15]. In the case of rice, the chromosomes are very small and the preparation of good chromosome samples has been relatively difficult. Thus the development of a reliable FISH method was deemed important for rice genetics and breeding.

In this paper, the development of an improved FISH method applicable to the location of genes and DNA sequences on rice chromosomes was outlined. Then the application of the method to localize rice DNA sequences was described. Finally, the characterization of *O. glaberrima* chromosomes by further improvement of the method was presented.

Development of an improved FISH method

A non-radioactive labeling method with biotin was developed and widely applied to the ISH method[22]. The 45S rDNA loci of rice were localized using biotinylated probes[6,12,13]. Due to the difficulty in preparing samples suitable for ISH and in the identification of rice chromosomes after ISH, it had been difficult to localize the genes in rice by ISH, although some results have been reported[6,10,24,25].

Three improvements in the fluorescence ISH (FISH) procedure enabled to overcome the difficulties and clear signals on rice chromosomes could be reproducibly obtained by using the improved method. Firstly, a set of post-treatments was introduced to remove the thin fluorescent layer which frequently covers the field after FISH treatment. Four post-treatments applied after sample preparation were as follows: (1) digestion of the polysaccharide layer originating mainly from the debris of the cell walls by the use of an enzymatic cocktail (2% Cellulase

Onozuka RS, 0.75% Macerozyme R-200, 0.15% Pectolyase Y-23, 0.5 mM EDTA, pH 4.2, 37°C, 30 min); (2) removal of chromosomal proteins by proteinase treatment (1 mg/ml Proteinase K, 37°C, 30 min); (3) elimination of the scattered cytoplasmic debris around the chromosomes by washing with 45% acetic acid (37°C, 5 min), which resulted in a considerable reduction of the noise caused by non-specific signals associated with the fragments of cellular debris; and (4) removal of ribonucleoproteins that are loosely associated with the surface of the metaphase chromosomes by RNase treatment (100 μl/ml, 37°C, 60 min).

Secondly, a modified thermal cycler was developed and was used throughout the denaturation process of the chromosome to facilitate hybridization of the probe DNA to the chromosomal DNA. The most critical step of FISH is the denaturation process of DNA. Usually this step lasts only a few minutes when the glass slide is dipped into a glutaraldehyde solution. The maintenance of a constant temperature for the solution is rather difficult after the glass slides are dipped in the solution since the heat capacity of glutaraldehyde is limited. The thermal cycler affords the most precise heat control since it was developed for the polymerase chain reaction. Thus the fluctuations caused by manual processing of the glass slides in and out of the solutions were practically eliminated.

Thirdly, an imaging method was introduced to analyze the FISH signal. Faint fluorescent signals could be enhanced and integrated into a chromosomal image. The effectiveness of imaging methods in plant chromosome research was first demonstrated in 1985 when a chromosome image analyzing system, CHIAS, was developed[3-5,7,9].

Variability in rDNA loci in the genus *Oryza* detected through the improved FISH method

The improved FISH method was applied to the physical mapping of 45S ribosomal RNA genes (45S

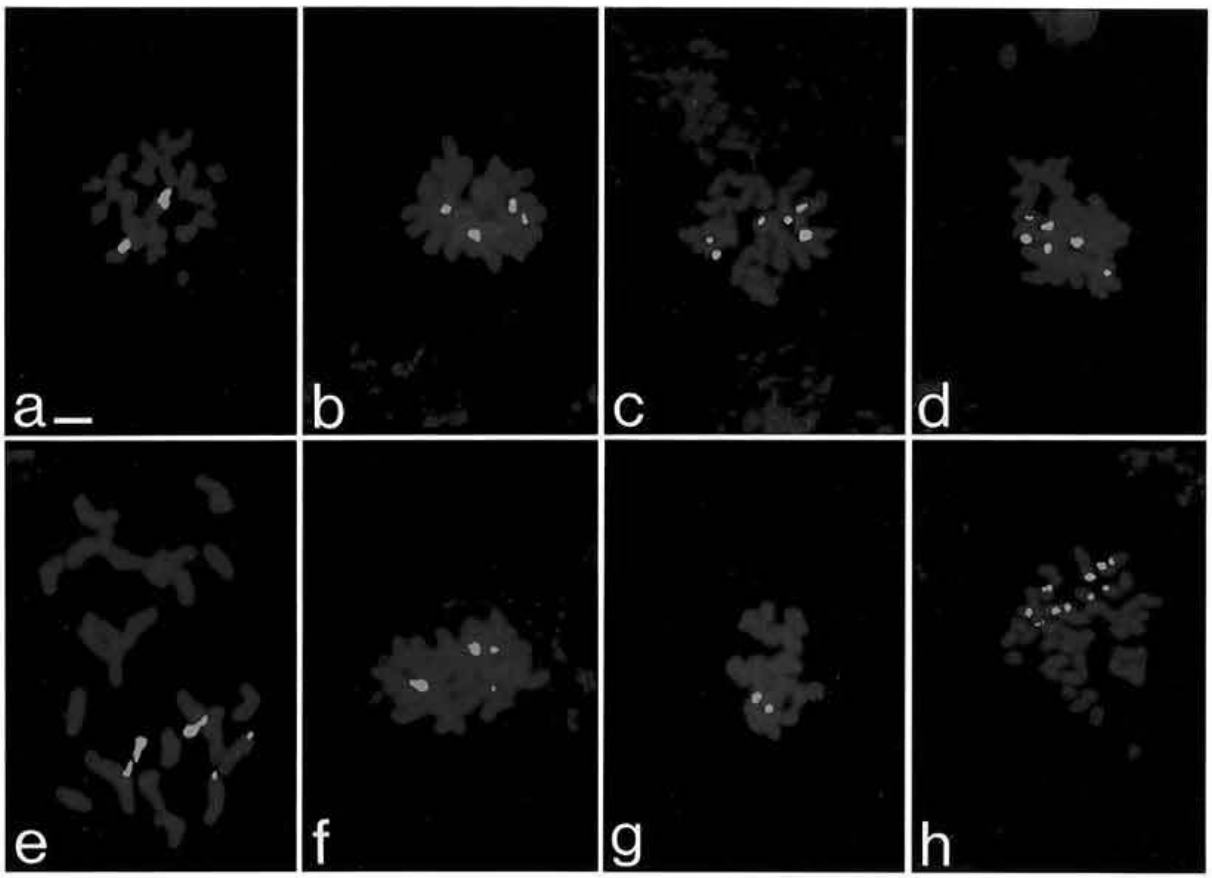

Plate 1. Fluorescent signals of 45S rDNA sites in seven rice species
a: *O. sativa* ssp. *japonica*, CH79, b: *O. sativa* ssp. *javanica*, Inakupa, c: *O. punctata*, d: *O. officinalis*, e: *O. eichingeri*, f: *O. australiensis*, g: *O. brachyantha*, h: *O. latifolia*. Scale bar indicates 3μm.

rDNA) in the genus *Oryza*. Variability in the number of 45S rDNA loci was detected among the eight diploid and one tetraploid species within the genus *Oryza* (Plate 1). Plate 1 shows that the 45S rDNA sites varied from one pair (Plate 1a, g), two pairs (Plate 1b, e, f), three pairs (Plate 1c, d) and five pairs (Plate 1h) within the chromosome complements.

A summary of the number of 45S rDNA loci detected by the FISH method in the nine *Oryza* species with the A, A^{gp}, B, C, E, F and CD genomes is given in Table 1. *Oryza* spp. with the F or A^{gp} genome showed one rDNA locus and species with C (*O. eichingeri*) or E genome showed two rDNA loci. *O. sativa* and *O. rufipogon*, both A genome species, were found to have either one or two rDNA loci. The species with B (*O. punctata*) or C (*O. officinalis*) genome were found to have three loci.

The NOR chromosome in the *Oryza* species with one NOR had already been identified as chromosome 9, on the basis of its morphology[8,9,16] and the presence of the 45S rDNA locus using the ISH method[6,10,12]. Another NOR chromosome was identified as chromosome 10[13]. Using the trisomic lines for chromosomes 9 and 10 in the current study, the signal intensity for each locus was found to be different. The locus on chromosome 9 exhibited a stronger signal intensity compared to that on chromosome 10. The third 45S rDNA locus has the weakest signal among the three 45S rDNA loci. Since there are no trisomic lines with the third locus in the wild species of *Oryza*, it is difficult to identify the chromosome bearing this 45S rDNA locus. The chromosome with the third 45S rDNA locus may be chromosome 11, according to preliminary observation.

Detection of the rice repeated sequences by the improved FISH method

In order to evaluate the effectiveness of the FISH method developed, rice DNA sequences with fewer nucleotide sequences were employed as the probe of FISH. The probes used in the experiment were tandem repeat sequences (TrsA) isolated from *O. sativa*. In rice, one family of tandem repeat sequences, which consists of a 355 bp sequence unit, has been identified in the various cultivars of *O. sativa*[2,21,26].

An analysis using ISH has indicated that the rice repeated sequences are located in the telomeric regions of the long or short arms of the chromosomes[25]. TrsA is considered to be localized in the subtelomeric sites based on the data of the sequence flanking TrsA[20]. Physical mapping of the TrsA using FISH

Table 1. Number of rDNA loci detected in cultivated rice (*O. sativa*) and the wild species studied

Species	Genome	Varietal group	Variety	Source and origin[a]	Number of rDNA loci
O. sativa	AA	*japonica*	Nipponbare	HNAES, Japan	1
			Aikoku	HNAES, Japan	1
			Tsushimaakamai	HNAES, Japan	1
			Tarizaohsen	HNAES, China	1
			Kouketsumochi	HNAES, China	2
			Ch78	NIG, China	1
			Ch79	NIG, China	1
		indica	Chinsurah Boro II	HNAES, India	2
			Kasalath	HNAES, India	2
			IR36	HNAES, India	2
		javanica	Ketan Nanga	HNAES, Indonesia	2
			Inakupa	NIG(221), Philippines	2
O. rufipogon	AA	annual type		NIG(W0106), India	2
		perennial type		NIG(W0149), India	2
		perennial type		NIG(W1944), China	1
O. glumaepathla	$A^{gp}A^{gp}$			NIG(W1192), Brazil	1
O. punctata	BB			NIG(W1582), Chad	3
O. officinalis	CC			NIG(W0002), Thailand	3
O. eichingeri	CC			NIG(W1521), Uganda	2
O. australiensis	EE			NIG(W1538), Australia	2
O. brachyantha	FF			NIG(W1401), Sierra Leone	1
O. latifolia	CCDD			NIG(W0019), Unknown	5

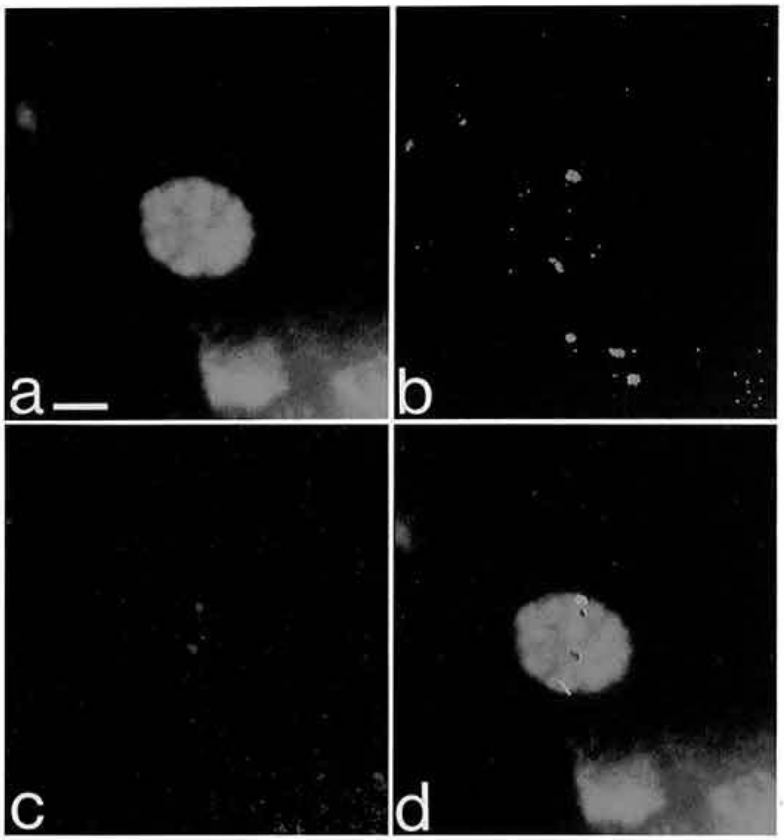

Plate 2. McFISH of *O. glaberrima* nuclei with simultaneous 5S rDNA and 17S rDNA probes
- a: DAPI image of interphase chromosomes obtained by UV excitation light.
- b: FITC image of the 5S rDNA signals in B excitation light.
- c: Rhodamine image of the 17S rDNA signals with G excitation light.
- d: Integration of the three images by imaging methods.

Scale bar indicates 5 μm.

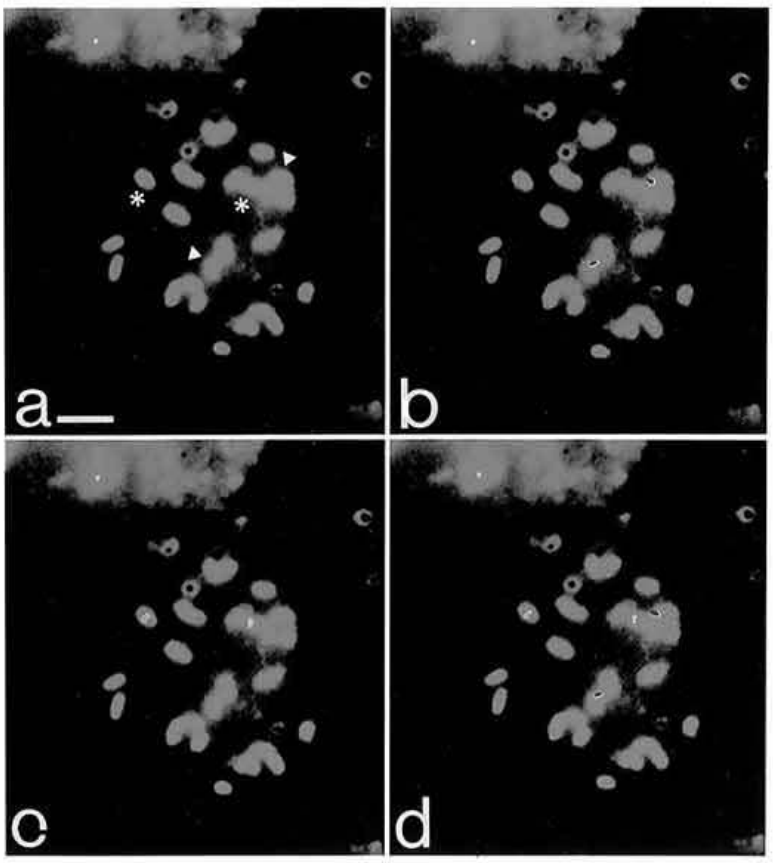

Plate 3. McFISH of *O. glaberrima* chromosomes with simultaneous 5S rDNA and 17S rDNA probes
- a: DAPI image of prometaphase chromosomes obtained by UV excitation light. Two pairs of chromosomes, chromosomes 9 and 11 are indicated by either solid arrowheads (▲) or asterisks(*), respectively.
- b: Rhodamine image of the 17S rDNA signals in G excitation light.
- c: FITC image of 5S rDNA signals in B excitation light.
- d: Integration of the three images by imaging methods.

Scale bar indicates 5 μm.

has revealed that the sequences are located in the distal end of the long arms of two to six chromosome pairs. Successful detection of a moderately repeated DNA sequence demonstrates the usefulness of the improved FISH method.

Characterization of rice chromosomes using a multicolor FISH method

Based on the FISH method, a multicolor FISH (McFISH) method was developed. Chromosomes of *O. glaberrima* were characterized by the McFISH method and conventional karyotyping. The McFISH method enabled to reveal the chromosomal organization through the detection of gene locations on the chromosomes.

As a result, McFISH simultaneously revealed the number of both 5S and 45S rDNA loci using biotin 5S rDNA (Bio-5S rDNA) and digoxigenin 17S rDNA (Dig-17S rDNA) as the probes (Plate 2). Plates 2a, b and c illustrate the DAPI(DNA binding fluorochrome, 4,6-diamidino-2-phenylindole dihydrochloride)-stained interphase chromosomes, the same image through a B excitation filter with a clear pair of signals of the 5S rDNA locus appearing within the complement, and the image through a G excitation filter showing a pair of signals of the 45S rDNA locus within the complement, respectively. *O. glaberrima* has only one locus for both the 5S and 45S rDNA as in the case of *O. sativa*[10,14]. Plate 2d shows the digital integration of the three different fluorescent images. Interphase chromosomes, the 5S, and 45S rDNA loci are visualized in blue, yellow, and red colors, respectively.

The 5S and 45S rDNA loci were mapped on rice chromosomes by FISH using Bio-5S rDNA and Dig-17S rDNA as the probes. The chromosome numbers of Giemsa-stained chromosomes were identified prior to FISH. Plate 3b shows the results of physical mapping of the 45S rDNA on the pair of satellite chromosomes. The satellite chromosomes indicated by arrowheads (Plate 3a) have been identified as the chromosomes corresponding to chromosome 9 of *O. sativa*[12]. A clear fluorescent signal was detected in the distal end of the short arm of chromosome 9 by FISH with the Dig-17S rDNA as the probe (Plate 3b). The asterisks indicate the DAPI-stained chromosomes with three heavily condensed regions along the chromosome, and it was confirmed that the *O. glaberrima* chromosomes corresponded to chromosome 11 of *O. sativa*[12]. The signal of 5S rDNA was located in the proximal region of the short arm of the chromosomes (Plate 3c). The doublet formation of the signals at the 5S rDNA locus indicates that the 5S rDNA has been replicated. Plate 3d shows a digital integration of the two signal images.

The 45S rDNA and 5S rDNA loci were physically mapped in the respective distal end of chromosome 9 and in the proximal region of the short arm of chromosome 11 as in the case of *O. sativa*. These results were in complete agreement with the loci on the rice RFLP map[23]. By the karyotype analysis of *O. glaberrima* chromosomes, it was concluded that *O. glaberrima* and *O. sativa* are quite similar to each other. Furthermore, the fact that *O. glaberrima* and *O. sativa* ssp. *japonica* show the same number and position of both the 5S rDNA and 45S rDNA loci in the genome, demonstrated the similarity of the chromosome organization.

Conclusion

The FISH method developed in this study has paved the way for developments in the molecular cytogenetics of rice. It enables to localize several genes on a rice chromosome. Distribution pattern of individual genes and repeated sequences should contribute to the analysis of the physical organization and genome dynamism of a chromosome and nucleus. Integration of the results accumulated by classical rice studies until now and the new data obtained by the technologies recently developed will enable to further promote research on rice genetics and breeding.

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