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# Monograph on Genus *Oryza*

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# Chromosome Research in Genus *Oryza*

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## INTRODUCTION

Rice, *Oryza sativa*: AA genome, composed of 24 chromosomes (n = 12) and 430 × 2 Mb total nucleotide length with about 1500 cM genetic length (Kurata et al., 1994b; Harushima et al., 1998). The private sector announced completion of full genome sequencing in early 2001. (http://www.myriad.com/pr/20010126.html). Follo-wing this, the international rice genome sequencing project reached a final goal for whole genome sequencing at the end of 2002. Until sequencing projects for all twelve chromosomes began, there were many chromosome studies which revealed basic morphological and genetic aspects in the latter half of the twentieth century. In the last decade, researchers have become able to use several new techniques, facilities, and molecular probes to show basic rice chromosome architecture. However, relationships between chromosome morphology/structure and molecular constitution, and between chromosome behavior and controlling mechanism are still wrapped in speculation. Now, chromosome research enters a new era for resolving the functional architecture of rice chromosomes and nuclei, as well as chromosome differentiation relating to evolutionary processes.

Entering this new chromosome research era, we should take a few minutes to review rice chromosome work in the last century. This chapter describes several key efforts in rice chromosome work done to date and reconsiders their meaning in the light of the new genome age. New trials for better understanding biological aspects of chromosome structure/function and applicable techniques for breeding programs using chromosomes are also discussed.

# KARYOTYPE ANALYSIS AND CHROMOSOME COMPARISON AMONG SPECIES

## Classic Studies

Rice, *Oryza sativa*, was first identified as having 2n=24 chromosome number in 1910 by Kuwada (1910). Following this observation, Rau (1929) reported that rice had five large, four middle, and three small-size chromosome pairs in its somatic metaphase. Nandi (1936, 1937) showed that somatic chromosomes ranged from 0.7 to 2.8 μm with the longest metacentric and two satellite chromosome pairs. However, Yasui (1941) observed 2.0 to 5.0 μm chromosomes having only one satellite chromosome of the 10th long subtelocentrics. Hu (1961) compared chromosome morphology among several *Oryza* species and reported that karyotypes were almost identical among those species, but chromosome size of *O. australiensiss* and *O. officinalis* were larger while that of *O. brachyantha* was smaller than other *Oryza* species. However, there were several discrepancies among these observations and chromosome morphology, which remained unclear until the 1970s because of its smallness and squash preparation method.

## Advances in Karyotype Analysis

Rice chromosome morphology began to be clarified in the 1970s and 1980s with the advance of chromosome preparation techniques in mitosis and meiosis (Kurata and Omura, 1978; Kurata et al., 1981). Rice chromosomes in somatic metaphase were so small in size; e.g. 0.8 to 0.4 μm from chromosome 1 to 12 in the late metaphase and 4.0-1.4 μm in early metaphase, so karyotype analysis was impossible at later metaphase stages. Instead, chromosomes in earlier stages, where chromosomes were 7-3 μm in pro-metaphase and 12-5 μm in late prophase were used. In addition, enzyme treatment for cell wall digestion appeared quite efficient to prepare well-spread clear chromosomes as shown in Figure 7.1. Thus, Kurata et al. (1978, 1981) succeeded in obtaining a clear karyotype with well characterized individual chromosomes of twelve chromosome pairs in early stages of mitosis. Figure 7.2 shows a karyotype with fine chromosome structure. However, further trials for C-banding and G-banding techniques were not always successful in obtaining reproducible results of banding patterns. Examples showing G-banding chromosomes and continuous changes of G-banding patterns according to chromosome condensation with cell cycle progression are shown in Figure 7.3. Following those works, a new idea to clarify chromosome staining pattern came into use through densitometric method analysis (Kamisugi et al., 1993). Applying image parameters to create specified characters on each chromosome, twelve rice chromosome images were established in prometaphase (Fig. 7.4).

The *O.sativa* karyotype was summarized as follows. Twelve pairs of chromosomes are composed of five metacentrics, five submetacentrics, and two subtelocentric chromosomes. Two chromosomes, the 7th and 9th were highly heterochromatic along the whole length. Centromere regions of most rice chromosomes were occupied with similar-size darkly stained heterochromatic blocks (Figs. 7.2 and 7.4). However, in several heterochromatic chromosomes, regions distal to centromeres are also stained darkly. The most remarkable

**Fig. 7.1.** Rice mitotic late prophase (a) and prometaphase (b) nuclei with chromosomes well spread by enzyme maceration/flame-drying metho d.

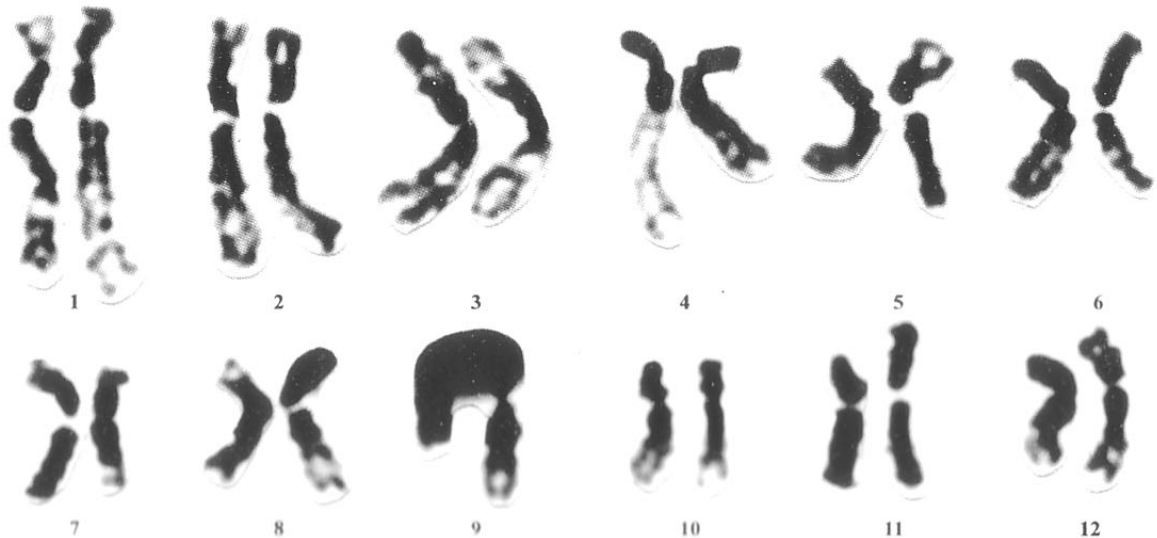



**Fig. 7.2.** A typical prometaphase karyotype of rice, *O. sativa* (2n = 24). The longest, Chromosome 1, is 8.4 μm and the shortest 3.3 μm.

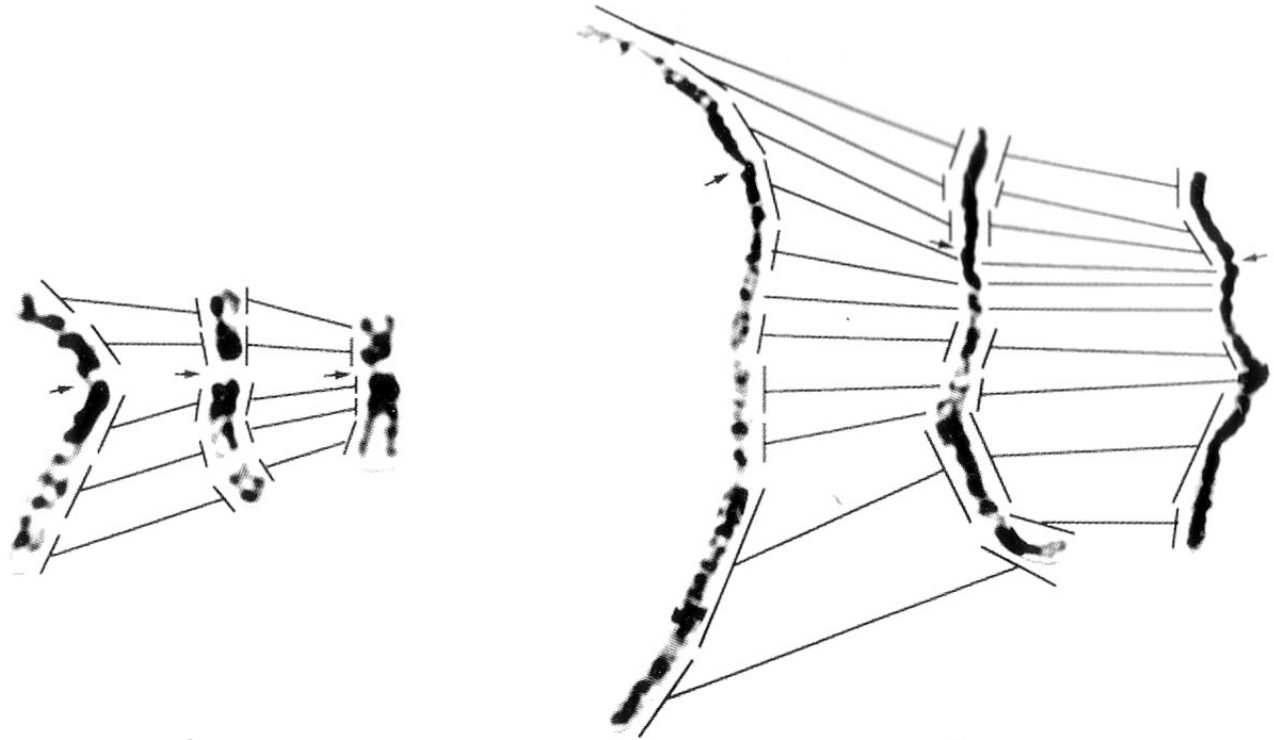



**Fig. 7.3.** Examples of G-band decrease in Chromosome 2 with progression from mitotic prophase to metaphase and chromomere decrease in meiotic prophase, pachytene in Chromosome 4. a - Chromosomes were contracted from late prophase (left), prometaphase (middle) to early metaphase (right) with detectable G-bands of 11, 8 and 5 respectively; b - Pachytene Chromosome 4 with fine chromomeres decreasing in number according to pachytene progression, from early (left), mid- (middle) to late (right) pachytene. Centromeres are indicated by arrows.

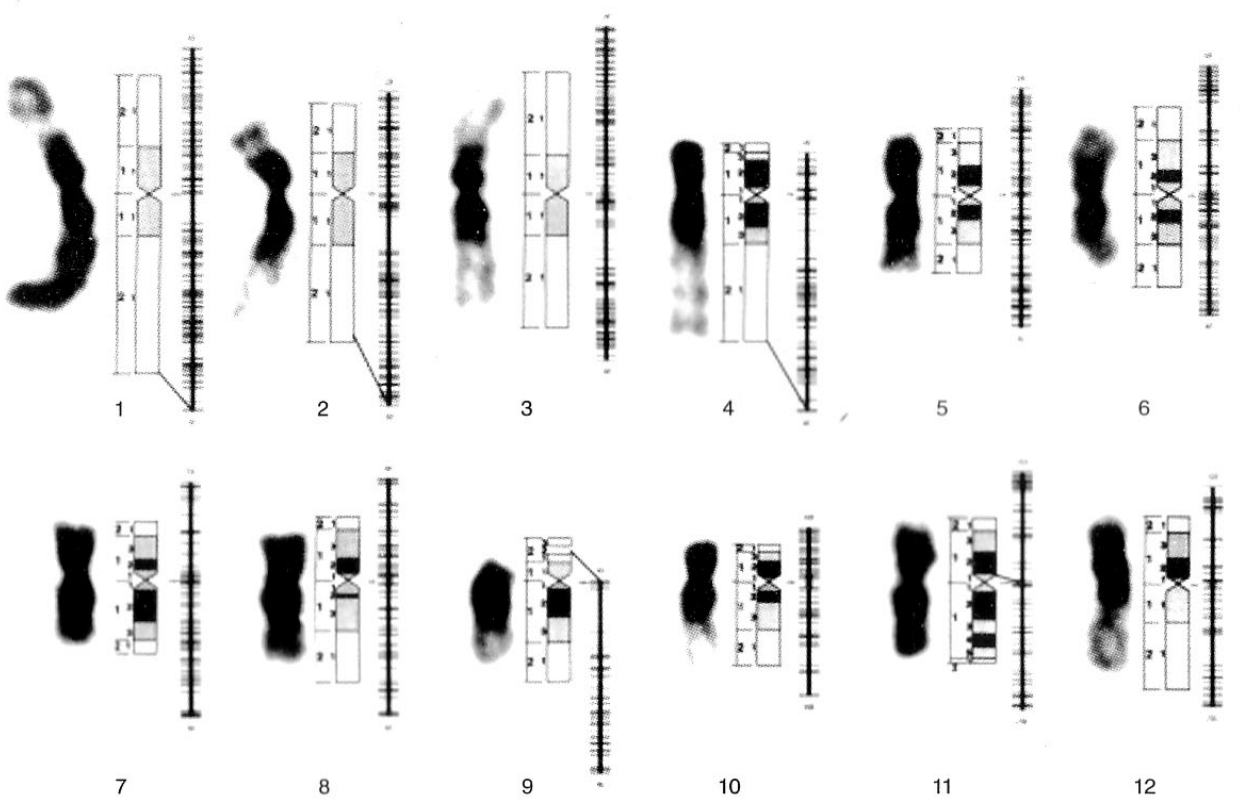



**Fig. 7.4.** Chromosome maps of rice (*O. sativa*, Japonica). The prometaphase chromosome karyotype shows a prominent condensation pattern, idiograms of prometaphase chromosomes, and linkage maps with molecular markers (from left to right). Lines in Chromosomes 1, 2, 4, 9 and 11 show positional relationships of known nucleotide sequences.

difference in chromosome character among species, even between Indica and Japonica rices, was position of the nuclear organizing region (NOR), which was known as secondary constriction. Japonica rice has a NOR in the distal portion of the short arm of chromosome 9, but in some Indica rices the NOR is located near the distal end of chromosome 10 short arm (Shishido et al., 2000). Details are given below.

## Pachytene Analysis

On the other hand, rice researchers have been using meiotic pachytene bivalent chromosomes to analyze cytological characteristics of chromosome structure. Pachytene chromosomes, which are one-half in number and longer in size compared to mitotic chromosomes, are very useful material for rice chromosome research. Analysis of pachytene bivalent chromosome morphology was first carried out by Shastry et al. (1960). Following this work, many reports of pachytene chromosomes for different strains and species were successively published by Sen (1963), Misra and Shastry (1967), and Chu (1967). These experiments showed similar results for all pachytene nuclei that the nucleus had twelve bivalent chromosomes ranging from 45 μm to 15 μm, from chromosome 1 to 12 respectively, with total length of 240-270 μm, and one or two large-size and two to five-small size nucleoli. Kurata et al. (1981) also applied new preparative techniques of chromosomes and obtained fine chromomere arrays on each of twelve bivalents. A complete set of pachytene bivalent chromosomes with fine chromomere pattern is shown in Figure 7.5. However, centromere positions, which were not necessarily visible on pachytene chromosomes occasionally, caused mistakes in chromosome discrimination. A trial to reveal centromere entities on pachytene bivalents was also applied successfully (Kurata and Omura, 1982).

High resolution mapping of chromomere patterns detected in late pachytene nuclei made it possible to clarify structural changes among different genome species. Karyotype analysis of four different genome species; *O. sativa* (AA genome), *O. punctata* (BB genome), *O. officinalis* (CC genome), and *O. brachyantha* (FF genome) could detect no obvious changes in mitotic prometaphase chromosome morphology (Kurata and Omura, 1982). Rapid chromosome contraction in prophase causes difficulty in comparing chromosomes, which are at the same stage of the cell cycle. Chromosomes with rapidly fusing chromomeres during pachytene stages are also shown in Fig. 7.3. Though there was the same problem of chromosome condensation in meiosis as mitosis, several small differences in chromomere patterns on some part of individual pachytene chromosomes were observed among *O. sativa* (AA genome), *O. punctata* (BB genome), and *O. brachyantha* (FF genome) (Kurata, 1986).

Causes of differences in chromosome structure and its relation to functional differentiation between genomes would be an interesting issue for future functional genomic studies in chromosome analysis. Recent advances in chromosome and genome research will provide several efficient approaches to do this kind of analysis in the chromosome field.


**Fig. 7.5.** Pachytene chromosome complement of *O. sativa*. Twelve paired bivalent chromosomes have fine chromomere arrays but unclear centromeres (indicated by arrows).

### Linkage Groups on Chromosomes and Chromosome Numbering

Pachytene chromosome analysis was also applied to identify extra chromosomes of 12 trisomic strains in *Indica* rice and to clarify relationships between chromosomes and genetic linkage groups (Khush et al., 1984). In advance of this work, Kurata et al. (1981) and Iwata et al. (1984) identified extra chromosomes in mitotic prometaphase cells of 12 trisomic series of *Japonica* rice together with genetic linkages of individual chromosomes. Although discrepancy in the relationship of some chromosome numbers with linkage groups was discerned between the two aforesaid experiments, reexamination by collaboration of two groups finally established the relationship between chromosomes and genetic linkage groups (reported by Khush, 1990). Designation of chromosomes having been established (Table 7.1), many genetic maps constructed to date, including the map with the highest maker density (Harushima et al., 1998), followed chromosome designation in 1990. Full genome sequencing projects are also carried out according to this chromosome number.

## GENOME AND CHROMOSOME SIZES

### Nuclear DNA Content and Genome Size of Genus *Oryza*

Nuclear DNA content and genome size of *Oryza* spp. have been determined using different methods of micro-photodensitometry (Katayama 1971, 1972) and flow-cytometry (Arumuganathan and Earle, 1991). Bennett et al. (1976, 1982, 1997) summarized and reported plant nuclear DNA content so that now all data are obtained via the Internet (http://www.rbgkew.org.uk/cval/database1.html). Nuclear DNA contents of 24 rice strains spanning 14 species have been recorded. Estimated genome size varies much among species from 392 to 1142 Mb. Genome size variability is also found even in the same species among researchers (e.g., 642 Mb and 946 Mb in *O. ridleyi*). These variations show methodological difficulty in obtaining reproducible data for genome size. Although there is considerable variation among reported data, the following may be said as a general tendency. First, amphidiploid species have more nuclear DNA content than diploid species. Second, B and C genome species have more nuclear DNA content than A and F genome species. These data suggest revision of previous genome sizes measured by micro-photodensitometry because genome size of B or C genome species had been estimated to be smaller than A genome species (Katayama, 1971, 1972). Third, *O. australiensis* with E genome has the largest genome size among diploid species.

Then, precise genome sizes of species in genus *Oryza* were examined using the same flow-cytometer with two internal controls of chicken red blood cells (CRBC) and cells of *O. sativa* cv. Nipponbare, which was the same variety used in the international rice genome sequencing project. Materials were also selected from those registered and maintained at the National Institute of Genetics, Japan and National Agricultural Research Center, Japan. Table 7.2 shows nuclear DNA contents of 12 diploid species with A, B, C, E, and F genome thus determined (Uozu et al., 1997). Genus *Oryza* shows much difference in genome size among its world wide species. There exists about a 2.7-fold variation in nuclear DNA content ranging from 0.72 pg/2C in *O. brachyantha* to 1.96 pg/2C in *O. australiensis*. Based on data obtained so far, the following two points are consistent with former results

**Table 7.1.** Final chromosome number agreement in 1990 (Khush/RGC)

| *Chromosome number* | *Karyotype and Trisomics* | | | *Iwata and Omura 1984* | *Linkage group Kinoshita/RGC, 1990* |
|---|---|---|---|---|---|
| | *Kurata et al., 1978, 1981* | *Wu and Chung, 1988* | *Khush et al., 1984* | | |
| 1 | K1 | 1 | 1 | O | III |
| 2 | K2 | 3 | 2 | N | X |
| 3 | K3 | 2 | 4 | M | XI+XII |
| 4 | K4 | 4 | 12 | E | II |
| 5 | K9 | 5 | 5 | L | VI+IX |
| 6 | K6 | 6 | 3 | B | I |
| 7 | K11 | 7(11) | 7 | F | IV |
| 8 | K7 | 9 | 8 | D | sug |
| 9 | K10 | 8 | 9 | H | V+VII |
| 10 | K12 | 10 | 10 | C | fgl |
| 11 | K8 | 11(7) | 11 | G | VIII |
| 12 | K5 | 12 | 6 | A | d-33 |

**Table 7.2.** Nuclear DNA content of twelve diploid rice species determined by flow cytometry

| *Species* | | *Genome* | *Accession** | *DNA content* | |
|---|---|---|---|---|---|
| | | | | *pg/2C* | *Mbp/1C*** |
| O. sativa | *Indica* cv. IR36 | AA | NARC | 0.93 | 449 |
| | *Japonica* cv. Nipponbare | AA | NARC | 0.91 | 438 |
| *O. glaberrima* | | AA | W0025 | 0.87 | 420 |
| *O. rufipogon* | | AA | W0120 | 0.95 | 459 |
| *O. longistaminata* | | AA | W0029 | 0.81 | 389 |
| *O. glumaepatula* | | AA | W1246 | 0.99 | 475 |
| *O. meridionalis* | | AA | W1625 | 1.02 | 493 |
| *O. punctata* | | BB | W1582 | 1.11 | 535 |
| *O. officinalis* | | CC | W0002 | 1.45 | 697 |
| *O. eichingeri* | | CC | W1521 | 1.47 | 709 |
| *O. australiensis* | | EE | W1538 | 1.96 | 946 |
| *O. brachyantha* | | FF | W1401 | 0.72 | 346 |

* NARC: National Agricultural Research Center. W numbered materials were provided by National Institute of Genetics.

** 1pg=965 Mbp.

accumulated by Bennett and Leitch (1997). Both B and C genome species have larger genome size than A and F genome species and *O. australiensis* has the largest genome. Moreover, *O. brachyantha* shows the smallest genome size among diploid rice species and C genome species have a much larger genome size than B genome species.

## Chromosome Size Variation in Genus *Oryza*

Chromosomes of eight diploid species of *O. sativa*, *Indica* and *Japonica* rice, *O. meridionalis*, *O. punctata*, *O. officinalis*, *O. eichingeri*, *O. australiensis*, and *O. brachyantha* were selected from 12 rice materials used in flow-cytometry. They were prepared by enzymatic maceration and air-drying (EMA, Fukui 1996), then stained with a Giemsa solution for detailed visual inspection. Figure 7.6 shows chromosomes of eight species under the same magnification. Chromosomes of *O. australiensis* were much larger than those of any other diploid species. Chromosomal regions especially at proximal regions of *O. australiensis* were densely stained or heterochromatic and the degree of chromosome condensation was high. On the other hand, chromosomes of *O. brachyantha* were smallest and visible heterochromatic regions were quite limited on their chromosomes. Average length of the *O. meridionalis* chromosomes was comparable to that of other A genome species except for conspicuous heterochromatic blocks at chromosome terminal regions.

These chromosomes were also subjected to image analyses to obtain two image parameters of total length and area. Both parameters show variation among diploid species just as with nuclear DNA content. *O. australiensis* shows the largest and *O. brachyantha* the smallest in both parameters, as suggested by their nuclear DNA content. Figure 7.7 shows nuclear DNA content against total length or total

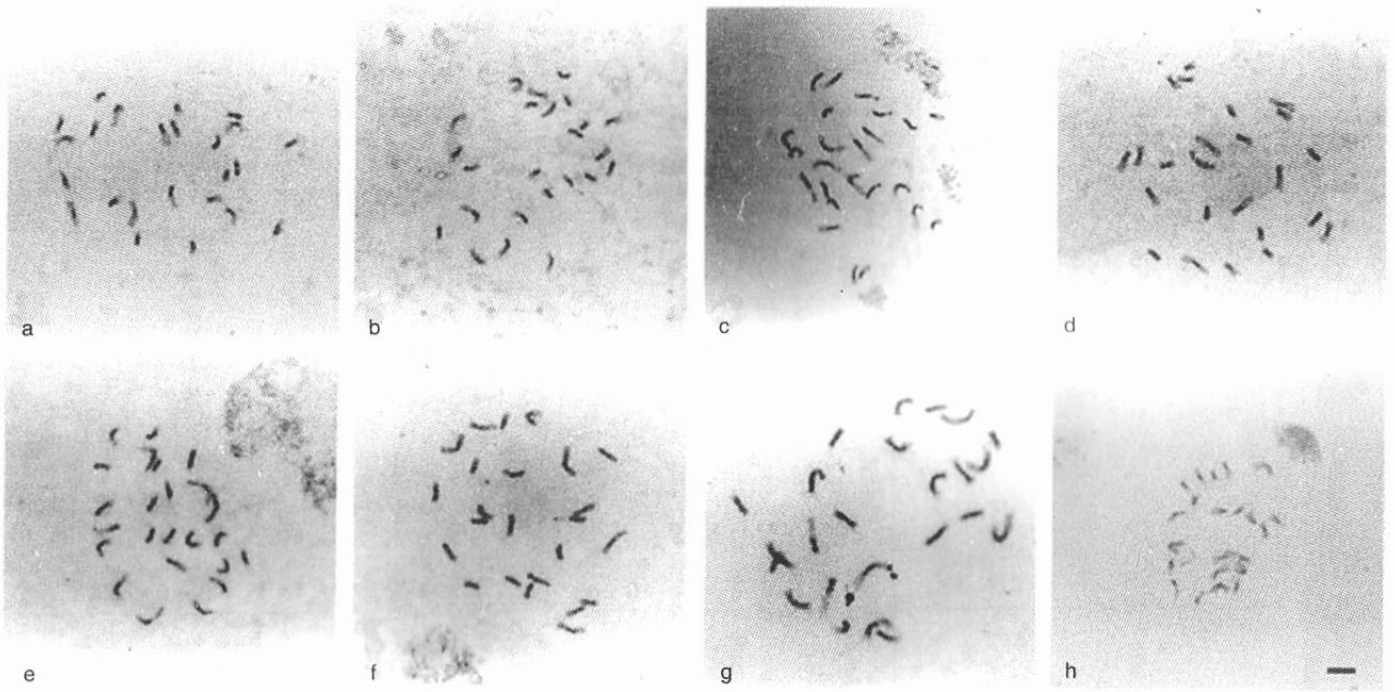



**Fig. 7.6.** Chromosome images of eight different diploid rice species prepared by the EMA method. a–*O. sativa*, Japonica (AA genome); b–*O. sativa* Indica (AA genome); c–*O. meridionalis* (AA genome); d–*O. punctaat* (BB genome); e–*O. officinalis* (CC genome); f–*O. eichingeri* (CC genome); g–*O. australiensis* (EE genome); h–*O. brachyantha* (FF genome). Bar, 5 μm.

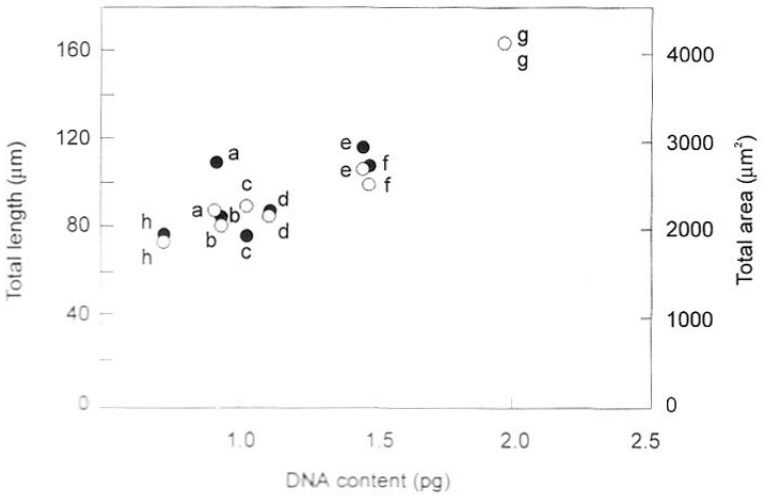



Fig. 7.7. Correlation between nuclear DNA content and total chromosomal length and area of eight diploid rice species. Open and solid circles represent total length and total area respectively. Letters a - h correspond to the species in Fig. 7.6.

area of chromosomes from each species. Statistical analyses on these two parameters show close relationships to nuclear DNA content. Correlation coefficients ($r$) are 0.939 and 0.927 between total length and nuclear DNA content and between total area and DNA content, respectively. Both are statistically significant at the 0.1% level. As a result, the classic chromosome length parameter is proven to give better estimation for rice genome size than chromosomal area (Uozu et al., 1997).

## PHYSICAL MAPPING OF SPECIFIC NUCLEOTIDE SEQUENCES ON RICE CHROMOSOMES

### Chromosome Map for Physical Mapping

For precise mapping of nucleotide sequences on defined chromosomal regions, it is a prerequisite to identify target chromosomes and to designate chromosome addresses before and after FISH. Condensation patterns showing uneven condensation along the chromosome, which appear on rice prometaphase chromosomes, are a good morphological characteristic to identify chromosomes and their addresses within chromosomes (Fukui and Mukai, 1988; Fukui and Iijima, 1991). Figure 7.4 depicts prometaphase chromosomes (Fukui and Iijima, 1991). Their maps are constructed by image analysis methods (Fukui and Iijima, 1991; Fukui, 1996), renumbered based on Khush (1990), and recombination maps based on molecular markers (Kurata et al., 1994, Rice Genome Program, Japan; (http://rgp.dna.affrc.go.jp/) of *O. sativa*, Japonica rice. Their positions are adjusted at the centromeric line and orientation of the linkage map is adjusted based on results reported by Singh et al. (1996).

Each rice chromosome demonstrates a unique condensation pattern at the mitotic prometaphase stage, which is a useful morphological characteristic in

identifying each rice chromosome (Kurata and Omura, 1978; Iijima et al., 1991). For example, rice Chromosome 1 is the largest submetacentric chromosome with large dispersed regions at distal regions of both its arms. Chromosome 2 is the third largest and submetacentric one, also with large dispersed regions at both distal regions. Chromosome 4 is a phantom-like chromosome of totally condensed short arm and a proximal region of long arm with a dispersed tail. Chromosome 11 shows a mostly condensed region with one and two heavily condensed centers on short and long arms respectively. It is now apparent that the condensation pattern at the prometaphase is ubiquitous among small plant chromosomes (Fukui et al., 2000) and serves as a good clue in identifying each chromosome of *Arabidopsis* (Ito et al., 2000), wild sugarcane (Ha et al., 1999), *Brassica* sp. (Fukui et al., 1998), *Lotus* sp. (Ito et al., 2000), and so on.

Rice prometaphase chromosomes were subjected to image analyses and three representative regions of heavily condensed regions (black bar), condensed regions (gray bar), and dispersed regions (blank bar) were determined by setting two gray values as thresholds which delineate heavily condensed and condensed regions, and condensed and dispersed regions. Each rice chromosome map was thus constructed based on 30 haploid rice chromosomal plates at the same stage within prometaphase. Addresses are also allocated to each region of the chromosome (Fukui and Iijima, 1991). The three largest chromosomes of Chromosomes 1, 2, and 3 have no heavily condensed regions. Chromosome 11 has three heavily condensed regions. Conspicuous differences are sometimes observed between the chromosome map and the linkage map. For example, 45S ribosomal RNA gene locates at the nucleolar organizing region (NOR) between the satellite and short arm and the NOR is observed cytologically. However, the NOR and satellite are not depicted in the case of the molecular linkage map. Although a resistance gene against bacterial leaf blight (*Xa-21*) is physically mapped at the condensed region between the two condensed regions on the long arm (11q1.3), the gene is mapped at the terminal region on the molecular linkage map (Ohmido et al., 1998). The same tendency has been reported in comparisons between chromosome maps and linkage maps of barley (Fukui and Kakeda, 1990; Kunzel et al., 2000) and rye (Gustafson et al., 1990). They proved that the recombination value is not even along the chromosome, but quite different from site to site. Terminal regions of chromosomes are characterized by especially higher recombination values than proximal regions.

## Development of *In-situ* Hybridization Methods in Rice

*In-situ* hybridization (ISH) method is a powerful tool for detecting visually specific nucleotide sequences on rice chromosomes. Rice ISH experiments were tried as early as the 1980s with the tritiated *rbcS* gene (Wu et al., 1986) and $^{125}$I-labeled rRNA (Fukui et al., 1987) as probes. Two signals at the end of the short arm of a pair of short chromosomes were clearly detected in the latter case. Three main obstacles in application of the ISH technique to rice chromosomes—small chromosome size, preparation difficulty, and chromosome identification difficulty—were mitigated by introducing an enzymatic maceration/flame-drying method (Kurata and Omura, 1978), enzymatic maceration/air-drying method (EMA, Fukui, 1996), and establishment of chromosome identification methods based on condensation pattern (Iijima et al., 1991). Fukui et al. (1994) reported variability in the number of rDNA loci among *Oryza* spp. using fluorescence ISH

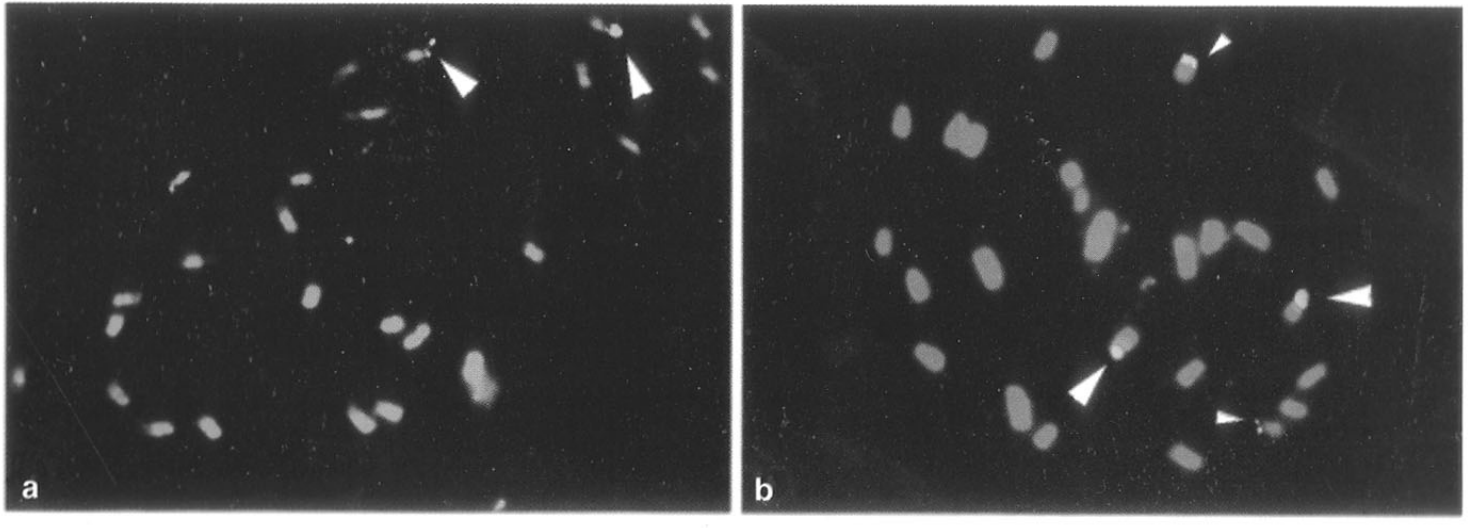



**Fig. 7.8.** Variation of 45S rDNA locus in *O. sativa* detected through FISH. Large and small arrowheads show Chromosomes 9 and 11 respectively. a - *O. sativa* Japonica; b - *O. sativa* Indica.

(FISH). Variation of rDNA loci from three to one among rice species was revealed. Even in the same species of *O. sativa*, variability in the number of rDNA loci was detected between Japonica and Indica rice with one and two loci respectively, as shown in Fig. 7.8. Table 7.3 summarizes all variations of 5S ribosomal RNA gene

**Table 7.3.** Location of rDNA clusters on chromosomes in rice spp.

| *Genome* | *Species* | *Chromosomes* | | | | |
|---|---|---|---|---|---|---|
| | | *4* | *7* | *9* | *10* | *11* |
| AA | *O. sativa, Japonica* | | | 45S | | 5S |
| AA | *O. sativa, Indica* | | | 45S | 45S | 5S |
| BB | *O. punctata* | | | 45S | | 5S |
| CC | *O. officinalis* | 45S | | 45S | | 5S, 45S |
| CC | *O. eichingeri* | 45S | | 45S | | 5S, 45S |
| EE | *O. australiensis* | | 5S | 45S | | |
| FF | *O. brachyantha* | | 5S | 45S | | |

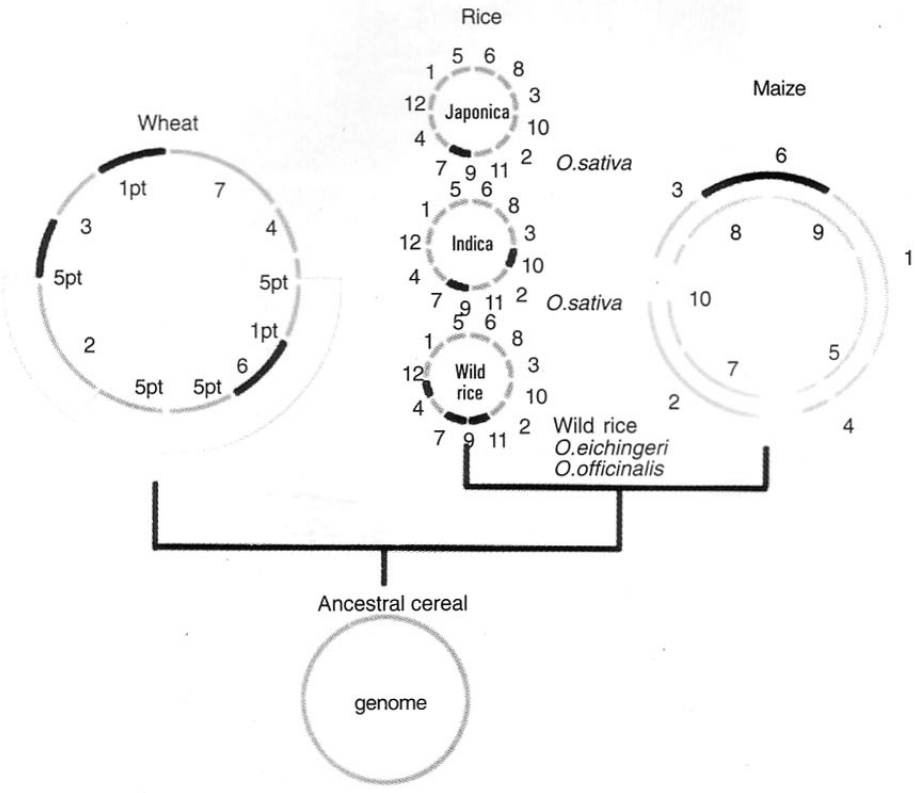



**Fig. 7.9.** Comparative analysis of cDNA loci in rice, maize and wheat. Numbers outside the bars forming circles are the chromosome number designated. Gray and black bars show the chromosomes with and without a 45S rDNA locus respectively.

(5S rDNA) loci and 45S rDNA loci detected in genus *Oryza*. Chromosomes harboring 45S rDNA loci are Chromosomes 4, 9, 10, and 11 and those with 5S rDNA are Chromosomes 7 and 11. Chromosome 9 is the common chromosome with a 45S rDNA locus throughout genus *Oryza*. Chromosome 10 has a 45S rDNA locus only in the case of *O. sativa*, Indica rice. The C genome species have three 45S rDNA loci on Chromosomes 4, 9, and 11. Chromosome 11 has two loci of 45S rDNA and 5S rDNA at close proximity as reported in the case of *Brassica* species (Fukui et al., 1998).

Moore et al. (1995a, b) developed an idea of collinearity for cereal genomes based on comparison of locations of the same genetic markers through different cereal genomes. Collinearity can be explained through evolution of cereal genomes and leads to the hypothesis of one single ancestral chromosome for all cereal genomes. Collinearity is found even in the fine-scale DNA marker order in rice and Triticeae genomes (Dunford et al., 1995). However, gene loci collinearity, characteristic among evolutionally close species, does not explain the case of 45S rDNA gene loci. Although one locus appearing on Chromosome 9 is common among rice species, the other locus on Chromosome 10 appearing in *O. sativa* (indica rice) is not found in other rice species. The third locus on Chromosome 4 appears only in the C genome species among rice species. These three loci, even the 45S rDNA locus on Chromosome 11, which is the common locus in genus *Oryza*, never appear at the expected chromosomal region in wheat, barley, or maize chromosomes predicted by gene loci collinearity among cereals as depicted in Fig. 7.9. The fact clearly indicates that the rDNA locus has a "nomad" nature, appropriately named by Dubcovsky and Dvorak (1995). The jumping nature of the rDNA locus among genomes has also been found in genus *Allium* (Schubert, 1984; Schubert and Wobus, 1985).

## Evaluation of Variability by FISH Methods using Repetitive Sequences

For genomic variability evaluation, repetitive sequences provide useful tools. Several rice repeat sequences have been cloned and characterized (De Kochko et al., 1991; Mawal et al., 1995; Zhao et al., 1989). A rice A genome specific tandem repeat sequence in *Oryza sativa*, Os48, has been cloned (Wu and Wu, 1987) and localized closely to telomeric sequences by ISH (Wu et al., 1991) and also by pulse-field gel electrophoresis (PFGE) (Wu and Tanksley, 1993). Ohtsubo et al. (1991) and Ohtsubo and Ohtsubo (1994) also cloned a rice A genome specific repeated sequence TrsA (Tandem repeat sequence A) from *O. sativa*, which has 355 bp unit sequence and also distributes specifically in rice A genome species. This TrsA has 90% sequence homology to Os48 and was used as the probe for FISH to detect intragenomic variability within three A genome species of rice. Figure 7.10 depicts FISH results using three different species within A genome: two, six and twelve TrsA sites were detected for *O. sativa* Japonica, *O. sativa* Indica and *O. meridionalis* respectively (Fukui et al., 2000). It is worth noting that the TrsA site shows variation within a species of *O. sativa* as in the case of the number of 45S rDNA loci; and Indica rice has a larger number of the TrsA sites and 45S rDNA loci than Japonica rice. All TrsA sites locate at the end of rice chromosomes except for one site that was detected at the interstitial region of Chromosome 4. TrsA locates at the end of short arms of the three largest chromosomes and locates at the end of

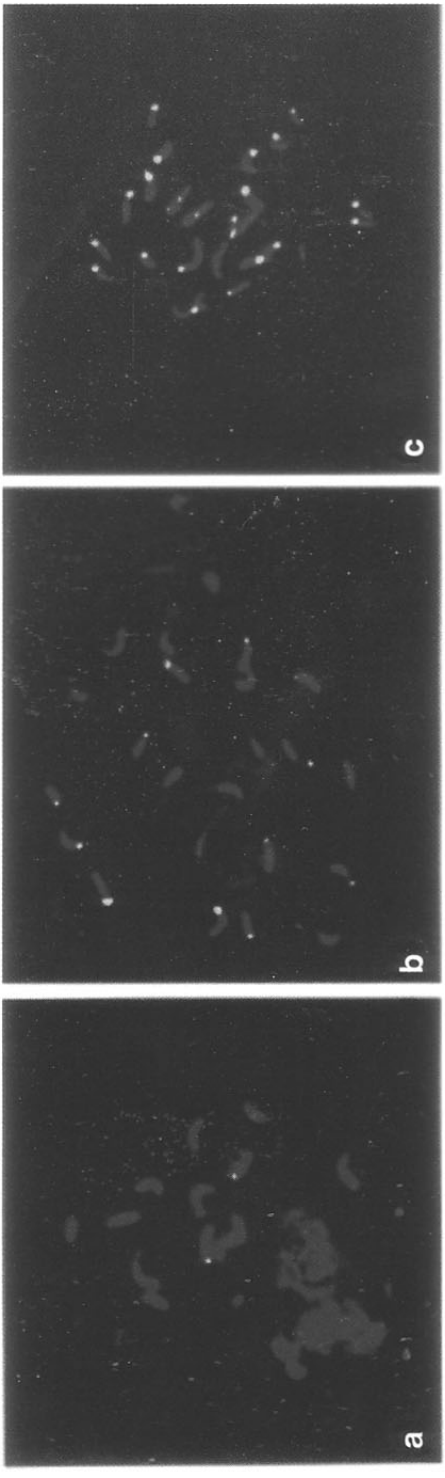



**Fig. 7.10.** Variation of TrsA sites among A genome species detected through FISH. a— *O. sativa* Japonica; b— *O. sativa* Indica; c— *O. meridionalis*.

long arms for the rest of smaller chromosomes in *O. meridionalis*. It is also interesting that TrsA site locations on chromosomes are complementary between Indica (Chromosomes 5, 7, 8, 9, 10, and 11) and Japonica rice (Chromosomes 6 and 12). *O. meridionalis*, which has twelve TrsA sites in a haploid chromosome set, shows telomeric condensation.

## Detection of Unique Sequences by FISH

Figure 7.11 depicts FISH experiment results from DNA probes with several sizes of unique nucleotide sequences ranging from 399 kb to 1.3 kb (Nakamura et al., 1997; Ohmido et al., 1998). The FISH experiment used three different probes. The largest probe is a YAC clone (Yeast artificial chromosome; YAC 2939 was kindly supplied by Drs. N. Kurata and T. Sasaki; Kurata et al., 1997) containing 399 kb rice genomic DNA. The second largest one is a BAC clone (Bacteria artificial chromosome, BAC 123 kindly supplied by Dr. S. Kawasaki) with 180 kb insert (Nakamura et al., 1997). A plasmid clone with 1.29 kb insert (kindly supplied by A. Saito, National Agriculture Research Center, MAFF, Japan) was also used. Clear doublet signals were detected at both chromatids of target rice chromosome(s) in all three cases, as shown in Figure 11. No significant nonspecific fluorescent noise was observed even in FISH using YAC and BAC clones as probes by simultaneous application of the two blocking DNAs of total rice sheared genomic DNAs and salmon sperm DNAs (Figs. 7.11a, 7.11b). Doublet signals from YAC and BAC were located at distal ends of dispersed regions of Chromosomes 1 (1q2.1) and 2 (2q2.1). Doublet appearance of the fluorescent signal guarantees genuine signals from hybridization sites. The same criterion was employed for signals from smaller probes. The YAC and BAC clones were thus physically mapped to addresses at 1q2.1 and 2q2.1. Figure 7.11c shows signals from the RFLP marker after FISH. The doublet signal from RFLP marker occurred at the end of Chromosome 4 (4q2.1). Occurrence frequencies of RFLP marker doublet signals were lower than those of YAC and BAC cases; the RFLP marker fluorescent signal had much weaker intensity than that from YAC and BAC clones. The probe PCR labeling method effectively enhanced fluorescent signals from the RFLP clone.

For larger size clones, difficulty in obtaining clear FISH signals lies in noise of nonspecific hybridization and/or hybridization with repetitive nucleotide sequences in the insert DNAs, which also hybridize to many other copies in certain chromosomal regions. A fifty-fold excess of total genomic DNA combined with probe DNA yielded high-contrast signals both for YAC- and BAC-FISH. Both concentrations of formamide and dextran sulfate significantly enhance fluorescent signals on occurrence of background noise and nonspecific hybridization of chromosomes. On the other hand, detection of faint fluorescence maintaining a high S/N ratio is essential for smaller probes derived from plasmid clones. No rice total genomic DNA was included in the hybridization mixture; only salmon sperm DNA was added as the blocking DNA. A cooled CCD camera is a useful device to detect faint signals at a low background noise level. Probe size is also essential to guarantee efficient access of the labeled probe fragments to complementary sequences in higher structure of a chromosome. Size 200 to 400 bp nucleotide sequences of labeled probes digested with DNase I resulted in a good approach of probe fragments to target regions.

As a result, rice FISH techniques now reach a similar level with human FISH, demonstrating reproducible physical mapping of unique sequences ranging from ca. 1 kb to 400 kb for rice chromosomes. The first generation ISH, based on usage of radioisotope labeling combined with micro-autoradiography, lasted from 1969 to the end of 1980. The second generation ISH, characterized by fluorescence detection of signals began in 1990. This FISH technology is especially effective to detecting mobile nucleotide sequences and/or genes existing as hemizygotic condition, both of which are difficult to detect by conventional molecular biological methods. FISH technology is technically satisfactory to detect single transposons and alien genes introduced to transgenic plants. In fact, a kind of retrotransposon (Hirochika, 2001) and several transgenes are successfully detected by FISH (Jin et al., 2001). By accumulation of data of physical localization of transgenes and/or transposons in rice chromosomes, it may be found that there are preferential integrated sites for transgenes, as in the case of oats (Leggett et al., 2000).

Now, third generation ISH is being developed, with detection of single nucleotide changes using rolling circle amplification (RCA) (Lizardi et al., 1998) and automatic detection of specific sequences by complementary nucleotide sequences with the aid of Rec A protein (Seong et al., 2000). These techniques are expected to be quickly introduced into rice chromosome research.

## More Versatile and High Resolution Mapping of Rice Nucleotide Sequences by FISH

There are two other technical points of ISH that need improvement other than the detection sensitivity improvement mentioned above. They are improvements of FISH efficiency by simultaneous detection of multiple probes and improvement of special resolution of nearby FISH signals. The former would reduce time and cost. The most time-consuming process in the FISH experiment is chromosome sample preparation. Thus, multiple probe FISH would save the number of good chromosome samples. The latter improvemment enable collection of information on exact localization of different nucleotide sequences and even estimation of the copy number of sequences providing the unit sequence length.

The technical basis allowing simultaneous detection of multiple probes is development of the fluorescent detection method and fluorescent dyes with different fluorescence spectra. Multicolor FISH (McFISH) in rice was first reported by Ohmido and Fukui (1997). This McFISH elucidated positional relationships between TrsA and telomere sequences. Although TrsA was physically mapped on the telomeric region of long arms of rice chromosomes, the distance of TrsA site relative to telomere sequences remained unknown. Thus, more detailed experiments using somatic chromosomes, interphase nuclei, pachytene chromosomes, and DNA fibers were carried out (Fig. 7.12; Ohmido et al., 2001). In this experiment, TrsA was labeled with digoxigenin and was detected by Texas red labeled antisheep IgG (red fluorescence). Telomere sequences were labeled with biotin and detected with fluorescein conjugated avidin (green fluorescence). Genomic and chromosomal DNA was stained with DAPI (blue fluorescence). As a result, TrsA sites were detected as red fluorescent signals and telomere sequences as green. The signals occupied the same regions at rice chromosomes or nuclei producing a white color by merging three primary colors of TrsA (red), telomere

(green), and DNA (blue) as shown in Figs. 7.12a and 7.12b. This indicated that figures TrsA and telomere sequences located in close vicinity as expected by the single probe FISH using each of the TrsA or telomere sequences.

Interphase mapping using interphase nuclei as targets gave higher resolution for localization of TrsA and telomere sequences. Figure 7.12b shows results of McFISH using TrsA and telomere sequences as probes to rice nuclei. TrsA signals were observed as partially overlapped ones in typical cases. It is understood that two signals were resolved partially, indicating that TrsA and telomere sequences were in tandem array on chromosome termini with and without a few intervening sequences between them. Telomere signals were usually included in TrsA signals, as shown in Fig. 7.12c, indicating that the TrsA domain is likely contributing a loop formation (Fig. 7.12d). Several new fluorescent dyes have been developed, such as Cy3, Cy5, and RGr, etc.; an easy labeling method of nucleotides was also reported with these dyes (Henegariu et al., 2000). They enhance FISH efficiency greatly in single experiments by detecting multiple probes simultaneously.

Extracted and extended on the surface of glass slides, DNA fibers have been used for the FISH target (Fransz et al., 1996). The copy number of TRG1, a subterminal repeat of tomato chromosomes, and its position relative to the telomere were estimated by measuring signal track length on DNA fibers after FISH. The EDF-FISH method was then applied to rice to estimate total length of TrsA and telomere sequences and their copy numbers on rice chromosomes (Ohmido et al., 2001). Figure 7.12e shows the result of EDF-FISH using TrsA (red signal tracks) and telomere sequences (one or two green signal dots). TrsA is directly connected with a few intervening sequences to a few green fluorescence tracks of telomere repeats. Signals from telomeric sequences generally appeared as a few dots only. Thus, there are few or no intervening sequences under the detection limit between TrsA and telomere sequences. Length of signal tracks was individually measured by image analysis methods for two chromosomes of *O. sativa* (Japonica rice) harboring TrsA at terminal regions, Chromosomes 6 and 12. The tandem repeat of TrsA on Chromosome 6 measured 25 mm on average, which corresponds to a molecular size of 82 kb (1 μm = 3.27 kb, Fransz et al., 1996) and a copy number of 231 (unit length 355 bp). Similarly, it was revealed that there are 682 copies of TrsA on Chromosome 12. Telomere sequences are 3 to 4 kb in average among twelve chromosomes (Ohmido et al., 2001).

The EDF-FISH is effective in estimating copy number of repetitive sequences. Then, difference in copy number of several repetitive sequences of TrsA, telomere sequences, 45S rDNA and 5S rDNA have been compared between Japonica rice (Nipponbare) and Indica rice (IR8) using EDF-FISH and ordinary chromosome FISH (Ohmido et al., 2000). Indica rice genome size is larger than that of Japonica rice by 37 Mb (significant at 1% level) and chosen repetitive sequences are different in their function, transcriptional nature, and contribution to chromosomal structure. Chromosomal FISH revealed that Indica rice has six sites of TrsA and 2 loci of 45S rDNA in a haploid chromosome set, and two TrsA sites and one 45S rDNA locus in the case of Japonica rice. However, it is not possible to detect differences in the 5S rDNA locus and telomere sequences because both Indica and Japonica rice have one 5S rDNA locus. Also, telomere sequences are ubiquitous at all chromosomal termini. The EDF-FISH reveals length, so the copy number of 5S rDNA and telomere sequences of Indica and Japonica rice are shown in Figure

7.13. Both repetitive sequences are much longer than those of Japonica rice (Ohmido et al., 2000). It is interesting that different randomly chosen repetitive sequences share the same tendency.

## USAGE OF REPETITIVE SEQUENCES FOR CHROMOSOME AND GENOME ANALYSIS

### Chromosome Painting Using Genomic DNA

Chromosome FISH and ISH using genomic DNA or genomic *in-situ* hybridization (GISH, Schwarzacher et al., 1989) are modified FISH methods using labeled total (genomic) DNAs as the probe. Because GISH utilizes all genome DNA including non-coding regions as the probe, the signal appears all over the chromosomal regions. This is why GISH is called a chromosome painting method, although some researchers defined the term "chromosome painting" as a method using unique repetitive and/or coding sequences which hybridize only to specific chromosome(s). Differences in repetitive sequences among chromosomes, even in differences in amount of repetitive sequences, lead directly to difference in fluorescence intensity appearing on chromosomes. As generally accepted, each genome has its unique constitution of repetitive sequences different from other genomes, so overall nucleotide sequence homology differs among different genomes. Nucleotide homology would be a measure for phylogenic distance between genomes. Three new genomes, G, H, and J were determined by genomic Southern hybridization based on this idea (Aggarwal et al., 1997). The GISH technique reveals overall sequential difference by different signal intensity emitted from the chromosome complement of identical genomes. Chromosomes belonging to different genomes show less signal intensity if different total genomic DNA is used as the probe. Thus, GISH is an effective tool for studying rice genomes from various points of view, as typically shown in the following study.

The rice D genome has been found only in tetraploid rice species in combination with the C genome as CCDD. Three tetraploid species with a CCDD genome, *O. latifolia*, *O. alta*, *O. grandiglumis*, are distributed in Central and South America (Vaughan, 1989). No diploid rice species in these regions has been classified as a D-genome species, while two diploid species, *O. officinalis* and *O. eichingeri*, with a C genome are distributed in Asia and Africa (Vaughan, 1989). Not only has no diploid D genome species been found in Central and South America, but also none has been found anywhere despite worldwide search and efforts to identify a D-genome species. There have been many views on possible diploid rice species with a D genome, the most common explanations being that the D genome has already become extinct and remains only in the tetraploid CCDD species (Jena and Kochert, 1991). Another is that the D genome is merely a variant of the C genome (Lu Baorong, Fudan Univ., China, pers. commun.), or that it has simply not yet been found. Thus, rice D genome identification and characterization has been a key issue in genetic, evolutionary, and phylogenetic studies of genus *Oryza* (Fukui et al., 1997).

The GISH method was applied to identify 24 chromosomes belonging to D genome among 48 chromosomes of amphidiploid species of *O. latifolia* with CCDD genome. Total DNA was isolated from diploid *O. officinalis* with C genome and labeled with biotin by the random primer labeling method. Then the labeled

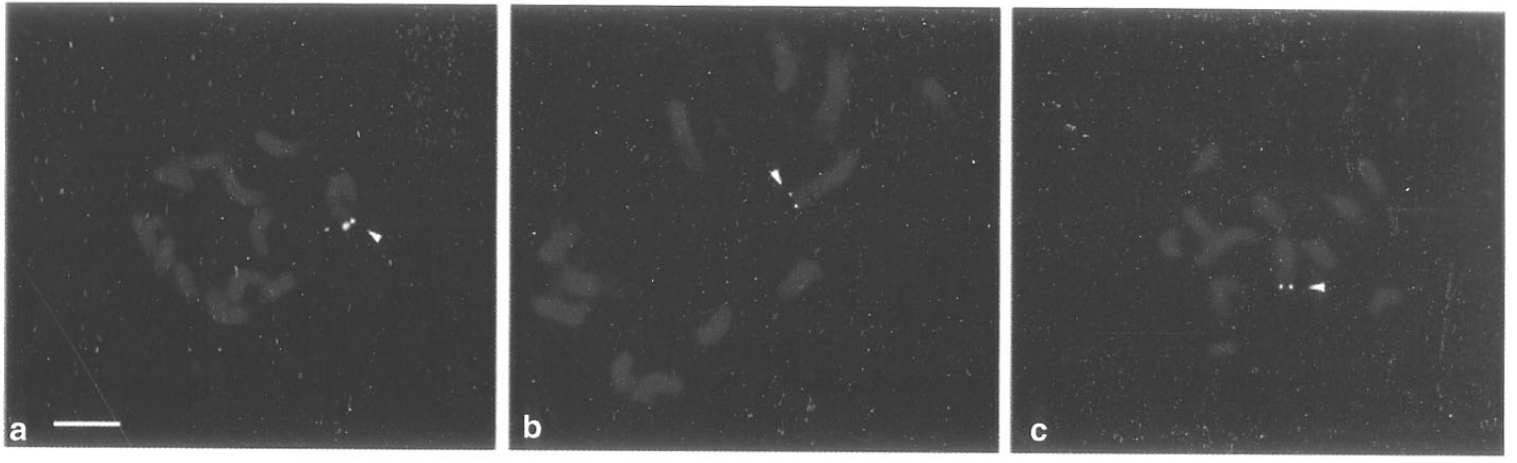



**Fig. 7.11.** Physical mapping of YAC, BAC and RFLP marker by FISH. a—Signals from YAC 2939; b—Signals from BAC 123; c—Signals from *Xnp247*. Bar, 5 μm.

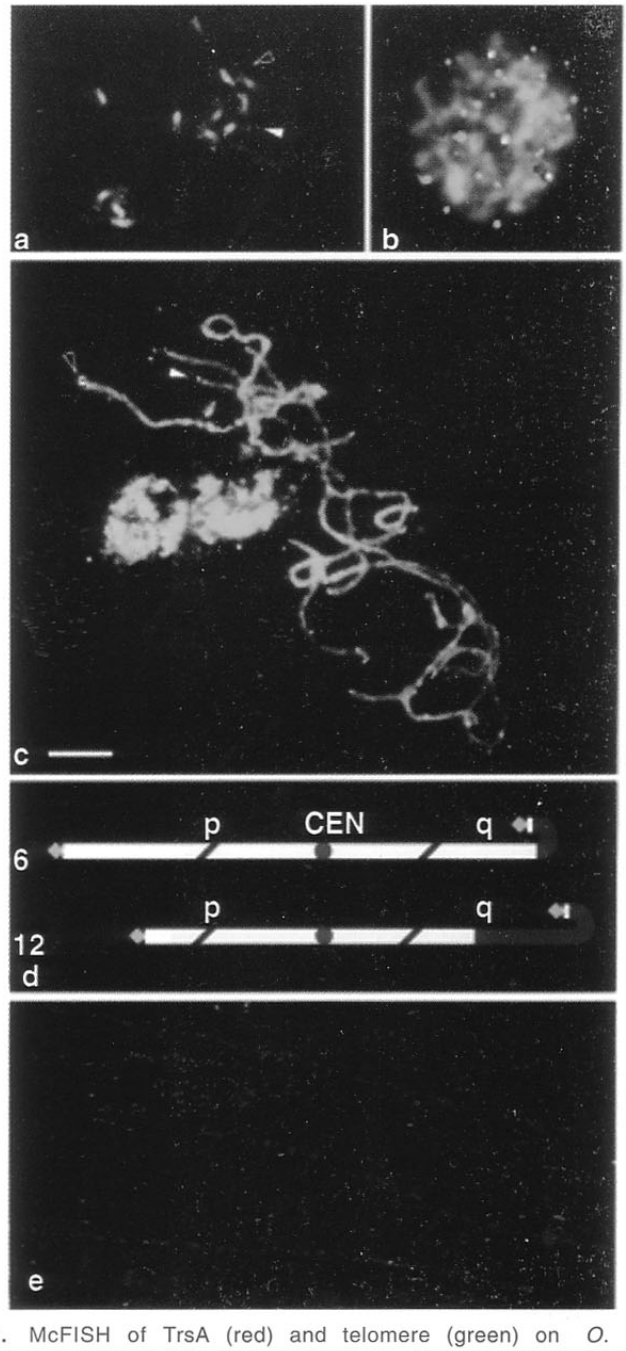



**Fig. 7.12.** McFISH of TrsA (red) and telomere (green) on *O. sativa* Japonica chromosomes, nuclei and DNA fibers isolated. a—Haploid prometaphase chromosomes; b—Diploid nucleus; c—Pachytene chromosomes; d—Model of chromosomal termini with TrsA. Cen, p, and q show centromeres, short and long arms respectively; e—Extended DNA fibers. Upper and lower tracks show Chromosomes 6 and 12, respectively. Bar, 10 μm.

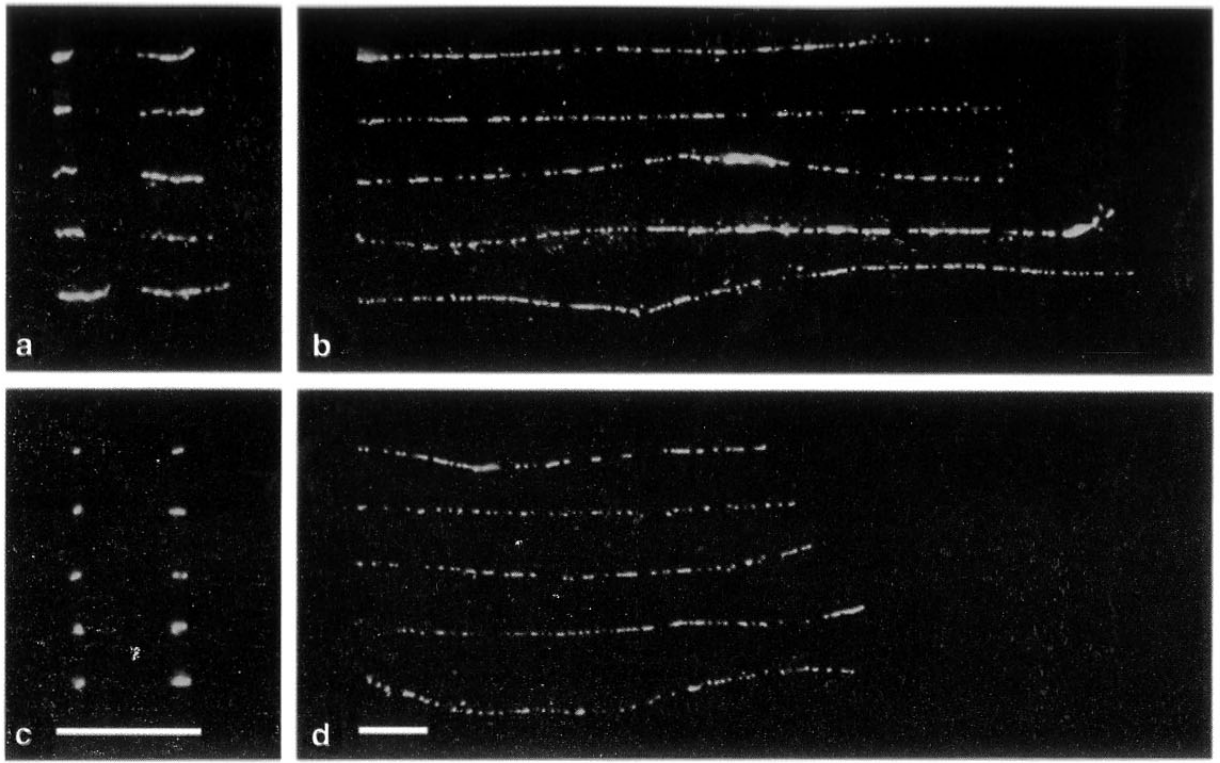



**Fig. 7.13.** Detection of telomere and TrsA regions on the extended DNA fibers by FISH. a and b—Signal tracks of telomere sequence (a, Indica; b, Japonica); c and d—Signal tracks of TrsA (c, Indica; d, Japonica). Bar, 10 μm.

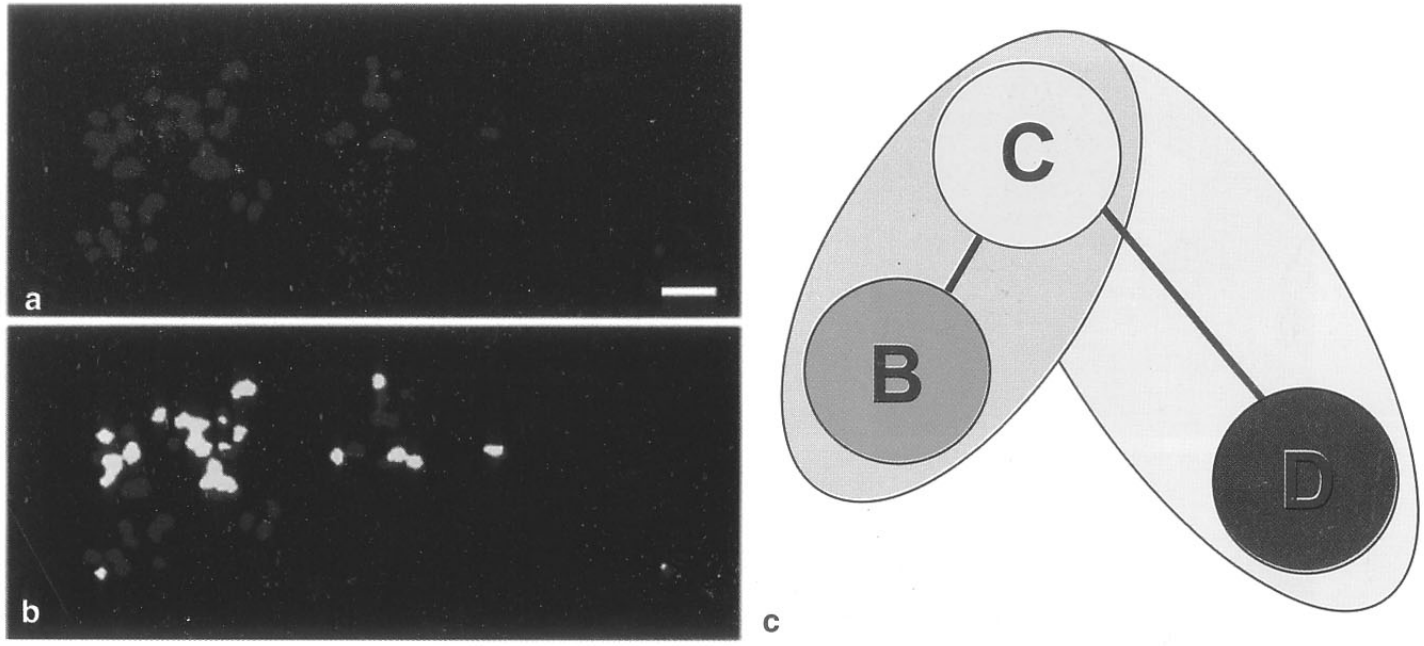



**Fig. 7.14.** GISH of *O. latifolia* (CCDD) chromosomes with total genomic DNA of CC genome species. a—Chromosomes counterstained with propidium iodide; b—Merged image with fluorescence signals; c—Schematic representation of phylogenetic relationship among the three genomes. Length between genomes in arbitrary units. Bar, 10 μm.

probe was hybridized to the chromosome samples of *O. latifolia* on a glass slide. Figure 7.14a shows propidium iodide stained chromosomes of *O. latifolia*. The 48 chromosomes at mid-metaphase were clearly counted. Figure 7.14b depicts a merged image of FITC (fluorescein isothiocyanate) signals after GISH and chromosome images. Twenty-four chromosomes were painted by clear fluorescence signals as a whole or partially, indicating that signal-tagged chromosomes were C genome chromosomes. The rest of the 24 chromosomes without a signal would be the D genome chromosomes. The image thresholding method was employed in this particular case because it was not possible to use DNA derived from the D genome diploid plant either for the probe or the competitive blocking DNA as in the usual GISH. It was also rather difficult to set a suitable threshold for *O. minuta* with BBCC genome when probing with the total genomic DNA isolated from the same C genome source, suggesting that phylogenetic distance between B and C genomes is closer than that of C and D genomes, as shown in Figure 7.14c. The result is consistent with phylogenetic analysis for genetic distance among rice genomes using chloroplast DNA (Dally and Second, 1990), ribosomal RNA gene spacer length (Cordesse et al.,1992), and genome specific RFLP markers (Jena and Kochert, 1991).

## Monitoring Chromosome Behavior after Wide Hybridization

Remote hybridization provides invaluable opportunities to introduce new characteristics from wild relatives to cultivated rice. To avoid unnecessary characteristics, it is a routine process to backcross parental cultivated rice until rice characteristics reach the sufficient level as the original cultivated rice except for the newly introduced trait. Overall genetic constitution of the remote hybrid is statistically estimated according to the number of backcrossings. However, it is empirically known that progeny show stability and similar characteristics at an unexpectedly early generation after remote hybridization between *O. sativa* and wild relatives. The mechanism of genetic transfer from wild relatives with different genomes to the cultivated A genome species after remote hybridization remains questionable because there is no recombination thought to occur between chromosomes belonging to different genomes.

Various remote hybridizations have been carried out in several countries. For example, hybrids between *O. sativa* and *O. australiensis* were obtained through embryo rescue in order to introduce resistant genes against brown planthopper and bacterial blight into *O sativa* (Multani et al., 1994) because remote hybridization usually shows hybrid sterility due to embryo abortion which may be caused by genomic differences between species employed.

Chromosome painting, or GISH, was employed to detect alien chromosomes within the $F_1$ and progenies. Because *O. australiensis* has the largest chromosomes among genus *Oryza*, it is a good clue for identifying *O. australiensis* chromosomes within a chromosome spread. Moreover, *O. australiensis* has specifically amplified repetitive sequences of *RIRE1* (Noma et al., 1996), although *O. punctata*, *O. officinalis*, and *O. eichingeri* also have *RIRE1* in a lesser amount (Uozu et al., 1997). This *RERE1* is a retrotransposon with sequence homology to *BARE1* isolated from barley.

Figure 7.15 depicts results of chromosome painting using *RIRE1*. All 24 *O. australiensis* chromosomes are clearly covered with fluorescence emitted from

*RIRE1* (Fig. 7.15a). On the other hand, 24 chromosomes of *O. sativa* show no fluorescence signals on any chromosome, indicating that there is almost no copy or no copy of *RIRE1* in its genome, as predicted by Southern hybridization using *RIRE1* as the probe (Fig. 7.15b; Noma et al.,1996). Now it is clear that *RIRE1* is a good marker DNA for discriminating *O. australiensis* chromosomes from those of *O. sativa*. Thus, the same monitoring method was applied to detect *O. australiensis* chromosomes in $F_1$ plants. Figure 7.15c shows 24 chromosomes of the $F_1$ plant after chromosome painting or GISH using *RIRE1* as the probe. Twelve chromosomes, larger in size than the remaining 12 chromosomes, demonstrate prominent fluorescent signals from *RIRE1*, indicating that these fluorescence-tagged chromosomes are E genome chromosomes. After some backcrossing, it was possible to obtain introgression lines of *O. sativa* with a few morphological characteristics introduced from *O. australiensis*. Figure 7.15d shows chromosome status of one progeny after backcrossing with 25 chromosomes. Some obvious morphological characteristics are introgressed into the *O. sativa*; however, there is no indication of existence of *O. australiensis* chromosomes, even a fragmental one. This fact would present existence of an interesting genetic transferring system under coexistence of different genomes within a cell. The system allows transfer of genetic material between chromosomes belonging to different genomes; transferred chromosome fragments are smaller than GISH technology detection limits. The latter is a quite small portion of chromosomal region transferred to the chromosome of the other genome as confirmed by usual molecular methods (Ishii et al., 1994).

## Multicolor Genomic *In-situ* Hybridization (McGISH)

As the FISH technique was improved from single gene detection to simultaneous multiple-gene detection, GISH was naturally developed for simultaneous detection of multiple genomes from single genome detection. Here we explain a case wherein McGISH is quite effective in analysis of genetic instability after somatic hybridization between *O. sativa* (Japonica) with AA genome and *O. punctata* with BBCC genome. Somatic hybridization is virtually the only major way available for generating hybrids between genetically distant plant species where an embryo rescue method is not available. However, the main problem of somatic hybrids and their progeny lies in genetic instability. It is quite often the case that somatic hybrids and their progenies show complete sterility. Various chromosome aberrations have been identified as the major cause of these genetic deficiencies.

Rice somatic hybrids were produced by fusion of protoplasts derived from diploid *O. sativa* (AA) and amphidiploid *O. punctata* (BBCC). Morphological characteristics of somatic hybrids showed complete sterility without exception. They also showed other variations in several traits different from the two parents and among somatic hybrid lines, such as short plant height, short panicle length, long flag leaf length, long spikelet length, unique isozyme band pattern, and delayed heading. Multicolor GISH (McGISH) was developed first in rice and employed to examine somatic hybrid chromosome constitution of temperate and tropical rice (Shishido et al., 1998).

GISH was employed to elucidate somatic hybrid chromosome distortion, which may be the cause of such morphological differences, using combinations of two different probes chosen among labeled three genomic DNAs of three diploid

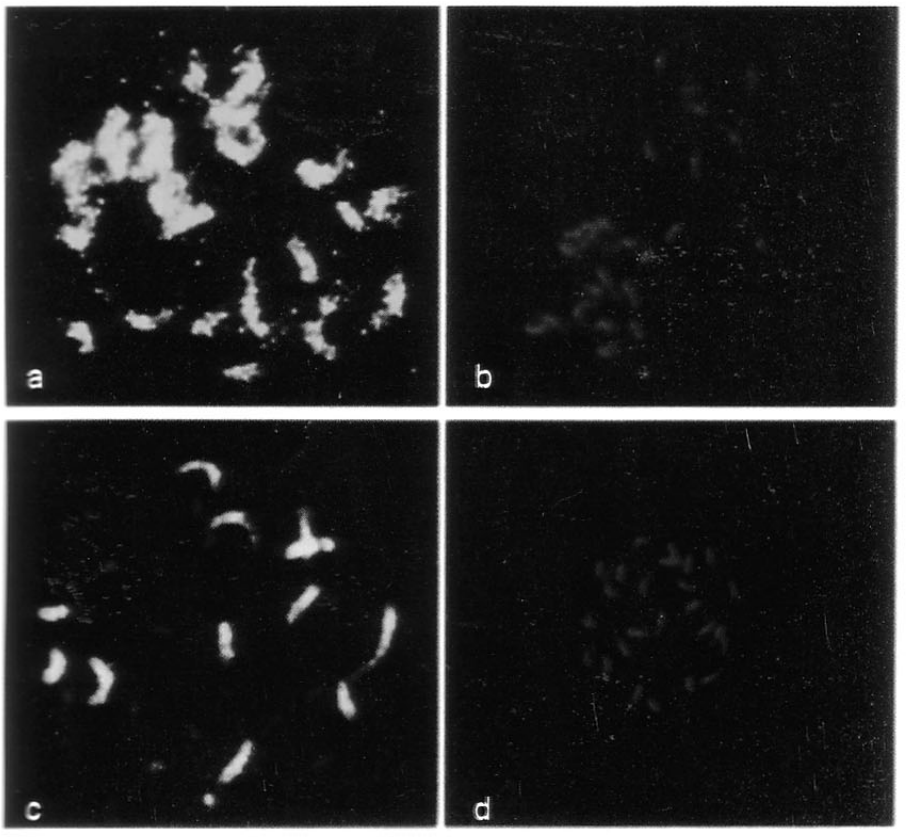



Fig. 7.15. Chromosome painting with a retrotransposon, *RIRE1*.
a—*O. australiensis;*
b—*O. sativa;*
c—$F_1$ between *O. australiensis* and *O. sativa;*
d—Monosomic alien addition line.

species, *O. sativa* (AA), diploid *O. punctata* (BB), and *O. officinalis* (CC). Figure 7.16 shows the somatic hybrid and results McGISH with the probe combination of A (detected and depicted by red fluorescence) and C genome (green) probes. Twenty-four chromosomes emit blue fluorescence, indicating chromosomes belonging to B genome. Because hybrids are produced by somatic hybridization between diploid and tetraploid species, the total chromosome number expected becomes 2n = 72. However, some reduction in chromosome number occurred; 65-72 chromosomes, were actually observed among cells from somatic hybrids. McGISH identified the genome of the lost chromosomes. Chromosomes of A genome were never eliminated from the complement and the expected chromosome number of 24 was always present in the nucleus of somatic hybrids (Fig. 7.16d). On the other hand, chromosomes belonging to B and C genomes (Fig. 7.16e) were frequently eliminated from the nucleus and B genome chromosomes

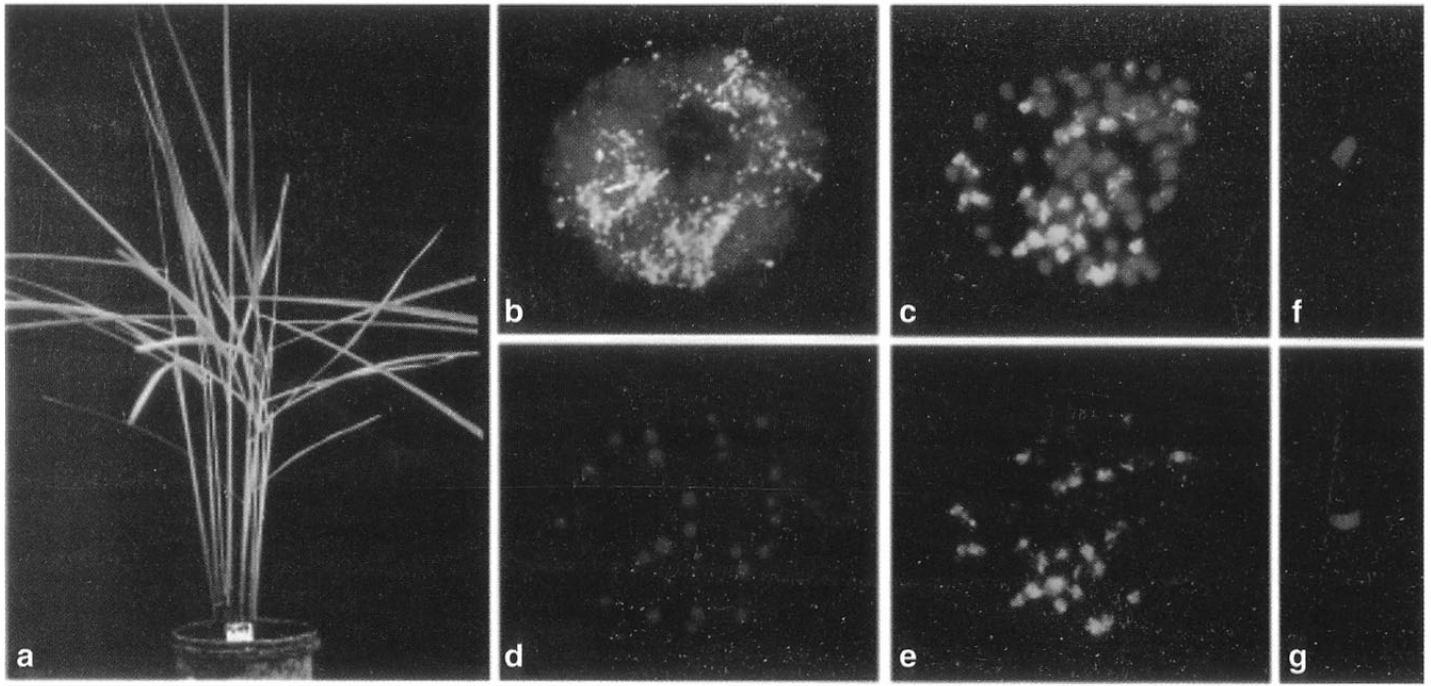



**Fig. 7.16.** Somatic hybrid (AABBCC, 2n = 65) and its chromosome constitution revealed by McFISH with genomic probes of A (red) and C (green). Chromosomes of B genome shown by blue color. a—Rice somatic hybrid; b—Nucleus; c—Chromosomes; d—A genome chromosomes; e—C genome chromosomes; f and g—Introgression of an A genome fragment into B genome chromosomes. Bar, 5 μm.

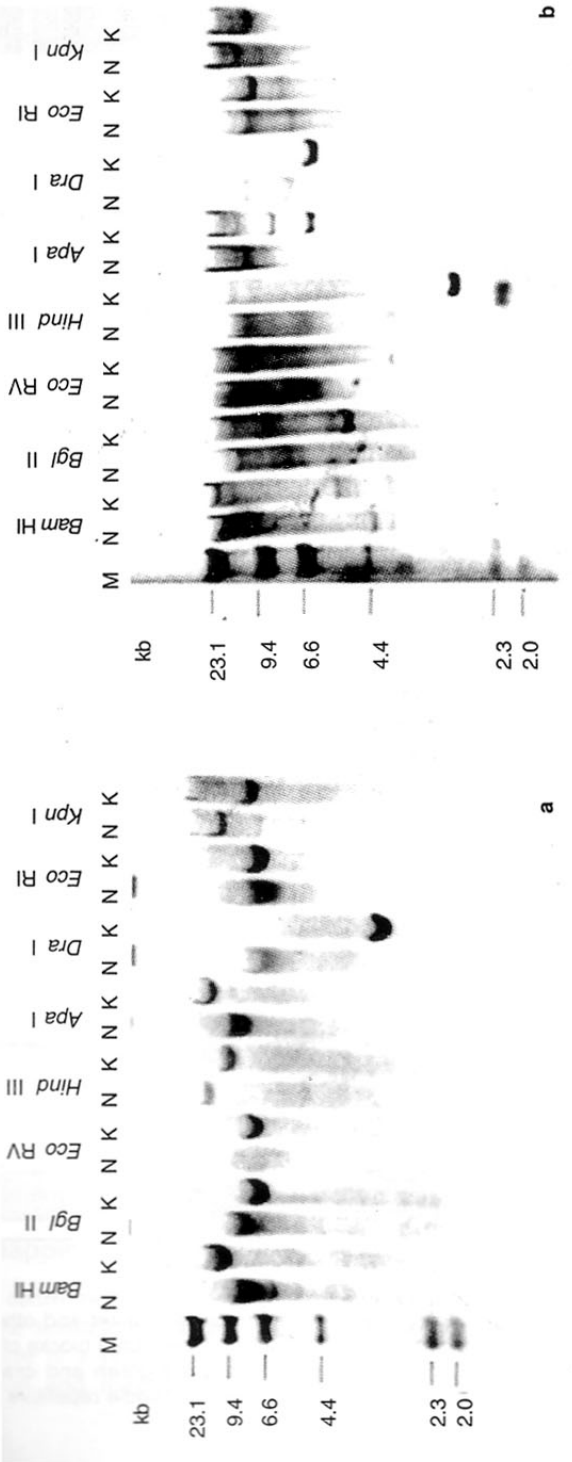



**Fig. 7.17.** Southern hybridization of *O.sativa* genomic DNA cut by eight enzymes and probed with telomere-associated sequences, TEL2 (a) and TEL3 (b). Hybridization bands over two and below about ten kilobase long are seen.

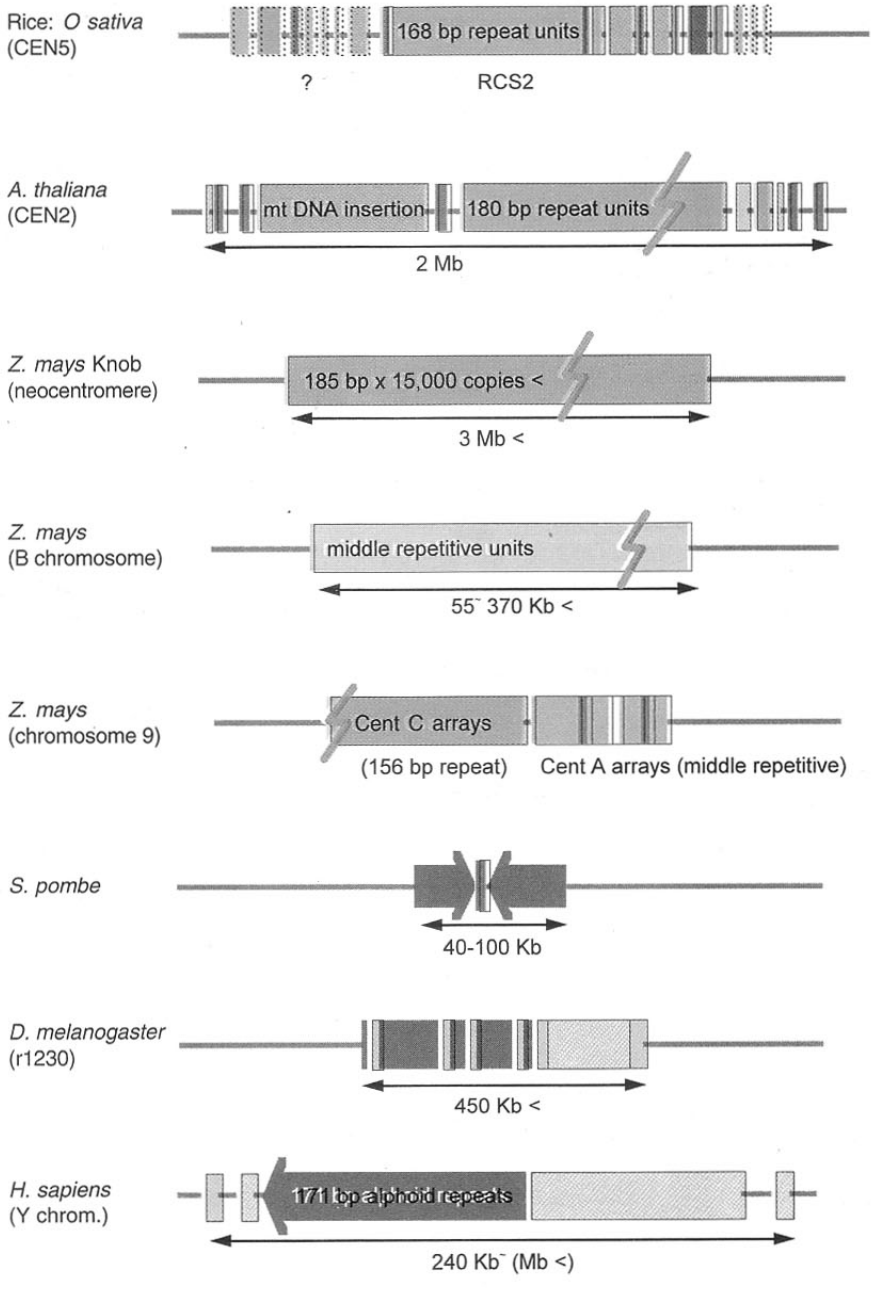



**Fig. 7.18.** Schematic figures of centromere structure of plant and other organisms. All centromeres have short tandem repeat (ca. 170 bp length unit) blocks of great length and transposon-like moderately repeated sequences. Light green and orange color blocks indicate short tandem repeat arrays, other color blocks middle repetitive sequences.

were more frequently eliminated than C genome chromosomes. Among the total 65 chromosomes appearing in Figures. 7.16, 16c, 24, 17, and 24 chromosomes were counted as A, B, and C genome chromosomes respectively. McGISH also revealed a genome-specific domain within the nucleus of the somatic hybrid (Fig. 7.16b). Thus, chromosome instability may be related to genome specific distribution within the nucleus. Furthermore, transferring of single chromosome fragments from A genome chromosomes to a pair of B genome chromosomes was observed (Figs. 7.16f, 7.16g). This fact presents fundamental data that exchange of genetic materials between chromosomes belonging to different genomes surely occurs in somatic hybrids and thus in hybrids consisting of different genomes.

In this experiment, retention of A genome chromosomes was commonly observed in somatic hybrids that grew well in the Hokkaido region, the northernmost part of Japan (latitude 41° – 46° N). The source of A genome is a cultivar bred in Hokkaido and that of BC genome is a wild relative distributed in the central part of Africa. It is likely that predominance of A genome chromosomes and reduction of chromosomes from *O. punctata* of the somatic hybrid may provide better adaptation for environmental conditions in Hokkaido (Shishido et al., 1998).

## CENTROMERE AND TELOMERE CHARACTERIZATION

### Telomeres as Chromosome Ends

Telomeres and centromeres are indispensable chromosome components both as structural key and functional importance in maintaining the chromosome entity itself. Rice telomere was shown to be composed of typical telomere tandem repeats of TTTAGGG hepta-nucleotides with various length from a few to below 10 kilobases in twelve chromosomes (Ashikawa et al., 1994) as shown by Southern hybridization analysis in Fig. 7.17 and also described above. Ashikawa et al. cloned four unique telomere associated sequences just connected to telomere repeats and mapped them on short arms of Chromosomes 1, 11, and 12 and on the long arm of Chromosome 3. Other telomere-associated sequences cloned and sequenced so far are all repetitive sequences. Wu et al. (1991) cloned another subtelomeric sequence from rice and hybridized on eight distal end regions of chromosome arms indicating it to be a repetitive sequence. Thus, rice telomere was revealed to have arrays of telomere tandem repeats of several to ten kilobases long, which were continued to the subtelomeric repeat sequences of other kinds. These telomeric and subtelomeric repeats were sometimes intervened with single-copy sequences unique to individual arms. This is a typical feature of chromosome ends observed in other organisms, also though positioning of telomere-associated unique sequences in other organisms very near to or at some distance from telomere repeats is not well known.

### Centromere Mapping on the Genetic Map

Cytologically, the centromere is clearly recognized as a primary constriction on the chromosome in the somatic prometaphase nucleus as shown in Figures 7.1 and 7.2. The centromere is a highly organized functional component necessary for faithful segregation and maintenance of chromosome structure and behavior

throughout the cell cycle. In rice, not all centromere positions are defined on genetic maps of twelve chromosomes. Centromere mapping was first tried by dosage analysis of RFLP markers using secondary trisomics and telotrisomics (Singh et al., 1996). In the second trial on a high-density genetic map, five centromeres could be mapped on a single locus or less than a 1 cM interval, but seven centromeres were not restricted to less than 1 cM (Harushima et al., 1998). Several loci, which carry multiple DNA markers, are located on almost centromere regions of twelve chromosomes and the loci with clustered markers could not be segregated in genetic mapping for over 180 plants $F_2$ population. The candidate centromere region composed of multiple DNA markers was often expanded to more than megabase length and distributed on several YAC clones (Umehara et al., 1996; Wang et al., 1996; Kurata et al., 1997). Physical lengths of individual centromeres were calculated for several chromosomes by YAC physical mapping and revealed to be at least one megabase (http://rpg.dna.afrc.go.jp/publicdata/physicalmap2001/YACall2001.html). Thus, similar to other organisms, rice centromeres were also suggested to be suppressed for their recombination over Mb length.

## Isolation of Centromere Repetitive Sequences

In most organisms, *Arabidopsis*, maize and several animals, centromeres have been proven to be composed of long stretches of short tandem repeats and various kinds of other repetitive sequences (Tyler-Smith and Floridia, 2000; Ananiev et al., 1998; Karpen and Allshire, 1997), as indicated in Figure 7.18. Rice has been searched for centromere-specific repetitive sequences and several kinds of repeat units identified, as shown in Table 7.4 together with other cereal plant centromere repetitive sequences. Aragón-Alcaide et al. (1996) first isolated a repetitive sequence, CCS1, commonly located on centromeres of most cereal plants, e.g. wheat, barley, maize, and rice. Dong et al. (1998) identified seven kinds of centromere-specific repeat elements from a BAC clone. Estimation of copy number in the genome showed six of them exist in 50 to 300 copies and the RCS2 sequence of 168 bp unit repeated over 5,000 copies in the rice genome. They also showed RCS2 was arranged as short tandem repeat blocks of various size up to 150 kb length. Another trial to isolate centromere repeat sequence by targeting the centromere protein binding box (CENP-B) was done and an RCE1 sequence, a 1.9 kb unit tandemly arrayed with intervening sequences, was isolated (Nonomura and Kurata, 1999). *In-situ* hybridization of RCE1 and RCS2 repetitive sequences showed the two sequences clearly located on the primary constricted regions with and without pericentromeric heterochromatic regions (Fig. 7.19). Differences in hybridization signal strength among chromosomes indicated the different number of those repetitive sequences from centromere to centromere.

On the other hand, retrotransposon studies in rice present evidence that most copies of gypsy-type retrotransposons, such as *RIRE3*, *RIRE7*, and *RIRE8*, are clustered on the centromeric heterochromatin, albeit truncated and modified (Kumekawa et al., 1999; Kumekawa et al., 2001; Nonomura and Kurata, 2001). These recent studies suggest that the rice centromere is composed of various repetitive sequences such as *RIRE3*, *RIRE7*, and *RIRE8*, lying in the range of at least one megabase. Now complexity and distribution of many repetitive sequences in the centromere comes into focus for characterizing and utilizing centromere structure and function.

**Table 7.4.** Centromere specific repetitive sequences in plants

| *Plant* | *Centromere repeat* | *Status* | *Total Length or copy number(cp)* | *Species specificity* | *Author & reference* |
|---|---|---|---|---|---|
| ***Arabidopsis*** | centromere repeat | | 400kb~1.4Mb (180 bp) | Arabi.specific | Round et al. Genome Res. 7 (1997) |
| **Cereal** | | | | | |
| **Maize** | B repeat (~1.4kbp) | short/middle | 55kb~Mb/CEN | maize specific | Alfenito & Birchler. Genetics. 135. (1993) |
| | neocent.rep. (185bp) | short tandem | ~9Mb | maize specific | Phelps/Birchler. Genome Res.7. (1997) |
| | CentC (156bp) | short tandem | 1000cp</CEN | maize specific | Ananiev et al. PNAS.95 (1998) |
| | CCS1/CentA | middle dispersed | | RT-LTR-like | Aragon-Alcaide et al. Chromosoma. 105 (1996) |
| ***B.sylvaticum*** (& cereals) | CCS1 | middle dispersed | | *Gramineae* (T3/gp)* | Aragon-Alcaide et al. Chromosoma. 105 (1996) |
| **Sorghum** | pSau3A9 (745bp) | middle dispersed | 81kb</CEN | *Gramineae* (T3/gp) | Jiang et al. PNAS.93 (1996) |
| | pSau3A10 (137bp) | short tandem | | sorghum specific | Miller et al. TAG.96 (1998) |
| **Rice** | CCS1/RCS1 | middle dispersed | | *Gramineae* (T3/gp) | Aragon-Alcaide et al. Chromosoma.105. (1996) |
| | RCS1 (877bp) | middle dispersed | 130cp | *Gramineae* (RT)** | Dong et al. PNAS.95 (1998) |
| | RCS2 (168bp) | short tandem | 6200cp (1Mb) | rice specific | Dong et al. PNAS.95 (1998) |
| | RCH1 (827bp) | middle dispersed | 53cp | *Gramineae* (RT) | Dong et al. PNAS.95 (1998) |
| | RCH2 (1.2kbp) | middle dispersed | 99cp | *Gramineae* (RT) | Dong et al. PNAS.95 (1998) |
| | RCH3 (1.3kbp) | middle dispersed | 67cp | *Gramineae* (RT) | Dong et al. PNAS.95 (1998) |
| | RCE1 (701bp) | middle dispersed | 287cp | *Bambusoideae* (T3/gp) | Dong et al. PNAS.95 (1998) |
| | RCE2 (2.7kbp) | middle dispersed | 305cp | *Gramineae* (RT) | Dong et al. PNAS.95 (1998) |
| | RCE1 (1.9kbp) | middle dispersed | 400~800cp | gypsy-type RT (*Bambusoideae*) | Nonomura and Kurata. MGG261 (1999)<br>Nonomura & Kurata. Chromosoma (2001) |
| | RIRE7 (7.6Kbp) | middle dispersed | 200~1700cp | gypsy-type RT | Kumekawa et al. MGG265 (2001) |
| | RIRE3 (8.0Kbp) | middle dispersed | several hundreds cp CEN specific | gypsy-type RT(pl K) | Kumekawa et al. MGG260 (1999)<br>Nonomura and Kurata. Chromosoma (2001) |

*T3/gp means T3/gypsy type retrotransposon sequence.
** RT means a kind of retrotransposon sequence.

## Structural Details of Rice Centromere: Case of Centromere 5

A detailed study of a centromere structure was carried out for Chromosome 5 and large centromere clones were isolated (Nonomura and Kurata, 2001). This helped to resolve an outline of Chromosome 5 centromere structure. Contig formation with YAC and BAC clones and genetic analysis has indicated that the Chromosome 5 (CEN5) centromere occupies more than 2 Mb. The CEN5 is composed of multiple arrays of various centromere repetitive sequences with a long RCS2 tandem repeat block in the center of the centromere. Distribution of major repetitive sequences has shown that the copy number of each repetitive member seemed highest proximate to RCS2 blocks and decreased towards distal ends of the centromere (Nonomura and Kurata, 2001). The maize centromere also showed a similar constitution centered with short tandem repeat block neighboring transposon-like sequences (Ananiev et al., 1998). Centromere-specific short tandem repeat units were always around 180 bp and specific to genus or species, whereas various retrotransposon-like sequences located distal to tandem repeat blocks seemed to be common for all members of species in a family (Table 7.4). All these repetitive sequence members together form centromeric heterochromatin on both sides of the primary constriction. These common features should suggest some functional and evolutional roles of repeated sequences and heterochromatin for centromere organization.

## Artificial Chromosome Construction and its Use

Rice artificial chromosome construction and production of a transgenic rice with it should be a challenging work not only for a basic chromosome study, but also for a breeding program. To construct an artificial chromosome, sequences having centromere and telomere functions are indispensable. Two such essential components of chromosomes were characterized, and at least some of those sequences have been cloned already. Several YAC clones carrying various centromere elements from CEN5 and telomere repeat sequence clones are good candidates for artificial chromosome construction. An example of constructing a rice artificial chromosome is shown schematically in Fig. 7.20. A YAC clone having both RCS2 repeat block and multiple copies of retrotransposons in its 380 kb DNA should be a promising candidate for centromere function. Another YAC vector having rice telomere repeats and plant selection markers has been constructed and used for retrofitting of the centromere YAC clone to form an artificial chromosome in a yeast cell (Kurata et al., 2001). Rice callus has a high potential for accepting foreign DNA and regenerating itself into a transformed plant capable of transmitting the introduced artificial chromosome to the next generation. This would provide a valuable opportunity to study genomic interaction between host chromosomes and an introduced artificial chromosome. It might also yield essential knowledge for generating novel plants using artificial chromosome technology.

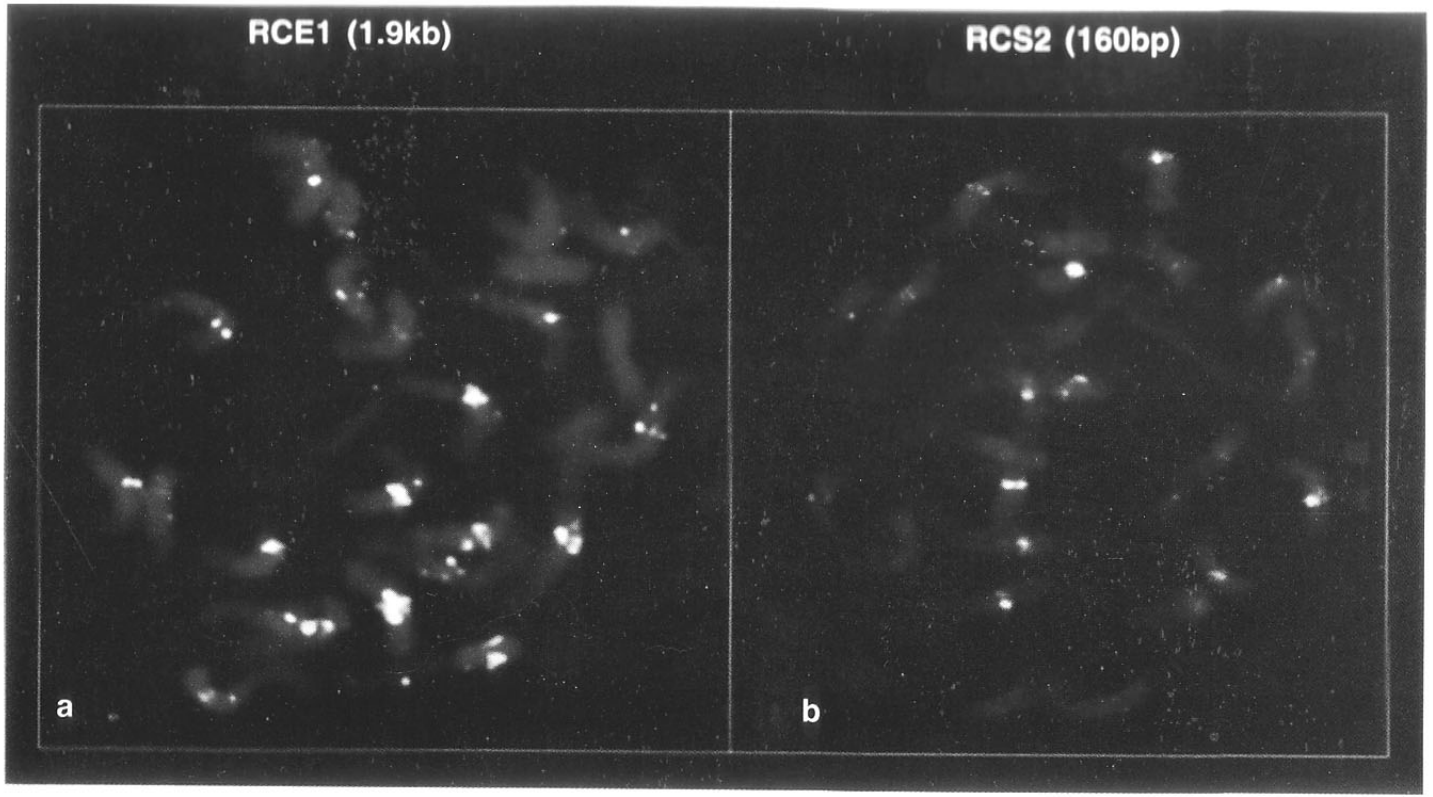
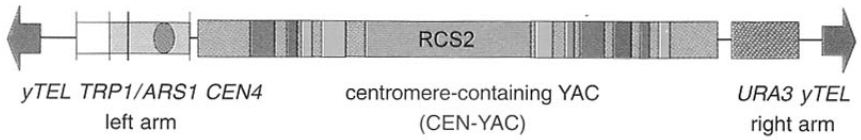


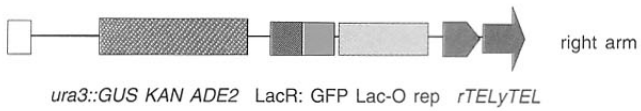

Fig. 7.19. FISH analysis using two centromere-specific repetitve sequences. RCE1(a) and RCS2 (b) are hybridized on almost all centromere regions showing various signal intensities.

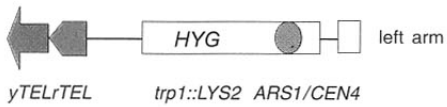
Construction and Introduction of a Rice Artificial Chromosome

1. Centromere isolation

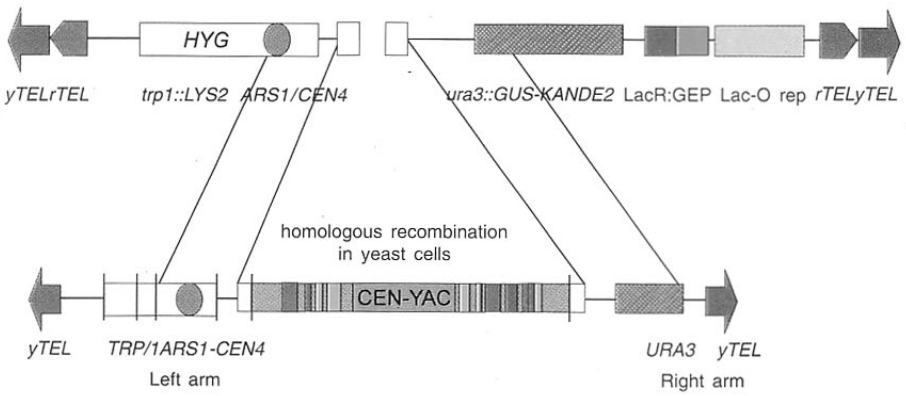



2. Construction of artificial chromosome arms for right and left ands




3. Retroffiting the CEN-YAC arms with artificial chromosome arms




4. Introduction of an artificial chromosome into rice cells by lipofection, PEG or biolistic method

5. Regeneration of transformed cells into plants carrying an artificial chromosomes

**Fig. 7.20.** Steps and strategies for constructing an artificial rice chromosome. An artificial chromosome should have a centromere sequence, a telomere sequence and rice selection marker genes. Reporter genes such as GUS (β-glucronidase) gene and GFP (green fluorescence protein) gene are very useful for tracing maintenance of introduced artificial chromosomes.

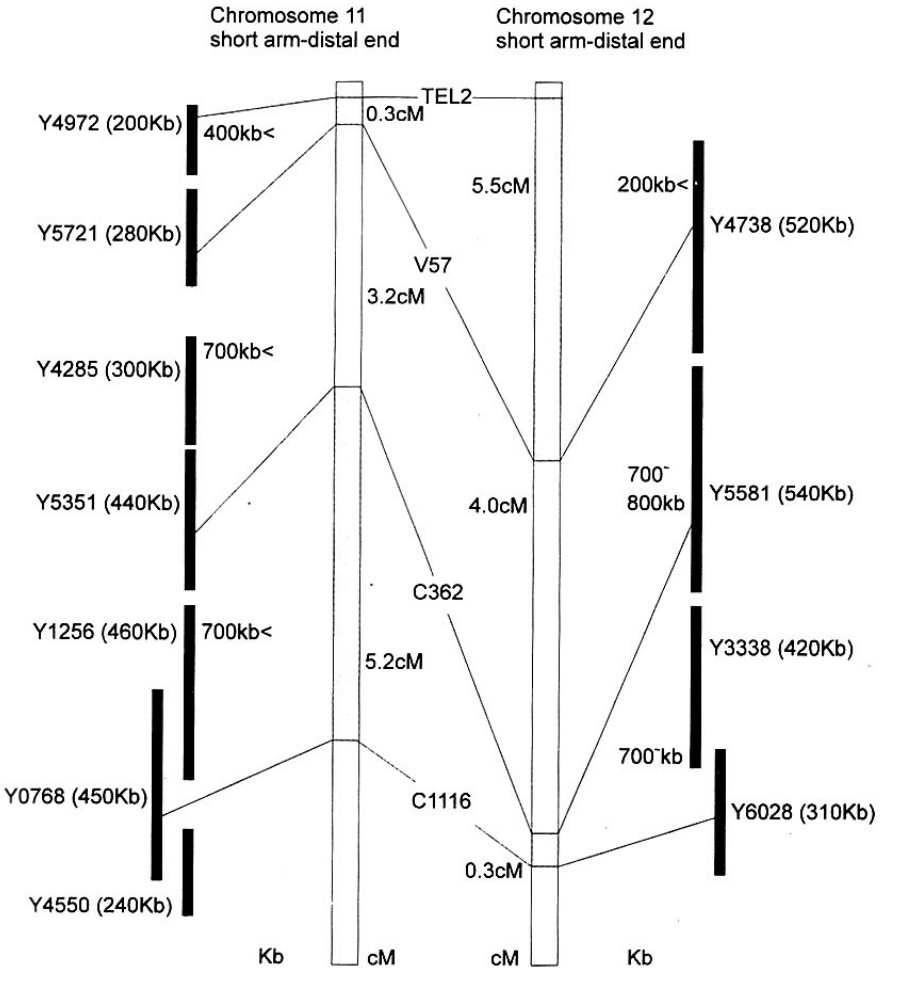



**Fig. 7.21.** Physical map of rice duplicated region on chromosomes 11S and 12S. Differences in ratio of genetic to physical distances shown by length comparison.

## RELATIONSHIPS BETWEEN RICE AND CEREAL CHROMOSOMES: EVOLUTIONAL ASPECTS

### Molecular Characterization of Rice and Other Cereal Chromosomes

Genome sizes of cereal plants differ: rice is 430 Mb, maize 3,000 Mb, barley 3,500 Mb, and wheat 7,000 Mb per genome (wheat, a hexaploid species with A, B, and D genomes, is 21,000 Mb in total). Rice chromosomes have longer euchromatic regions and relatively shorter heterochromatic regions compared to other cereal chromosomes. The higher the ratio of heterochromatin to euchromatin, the larger

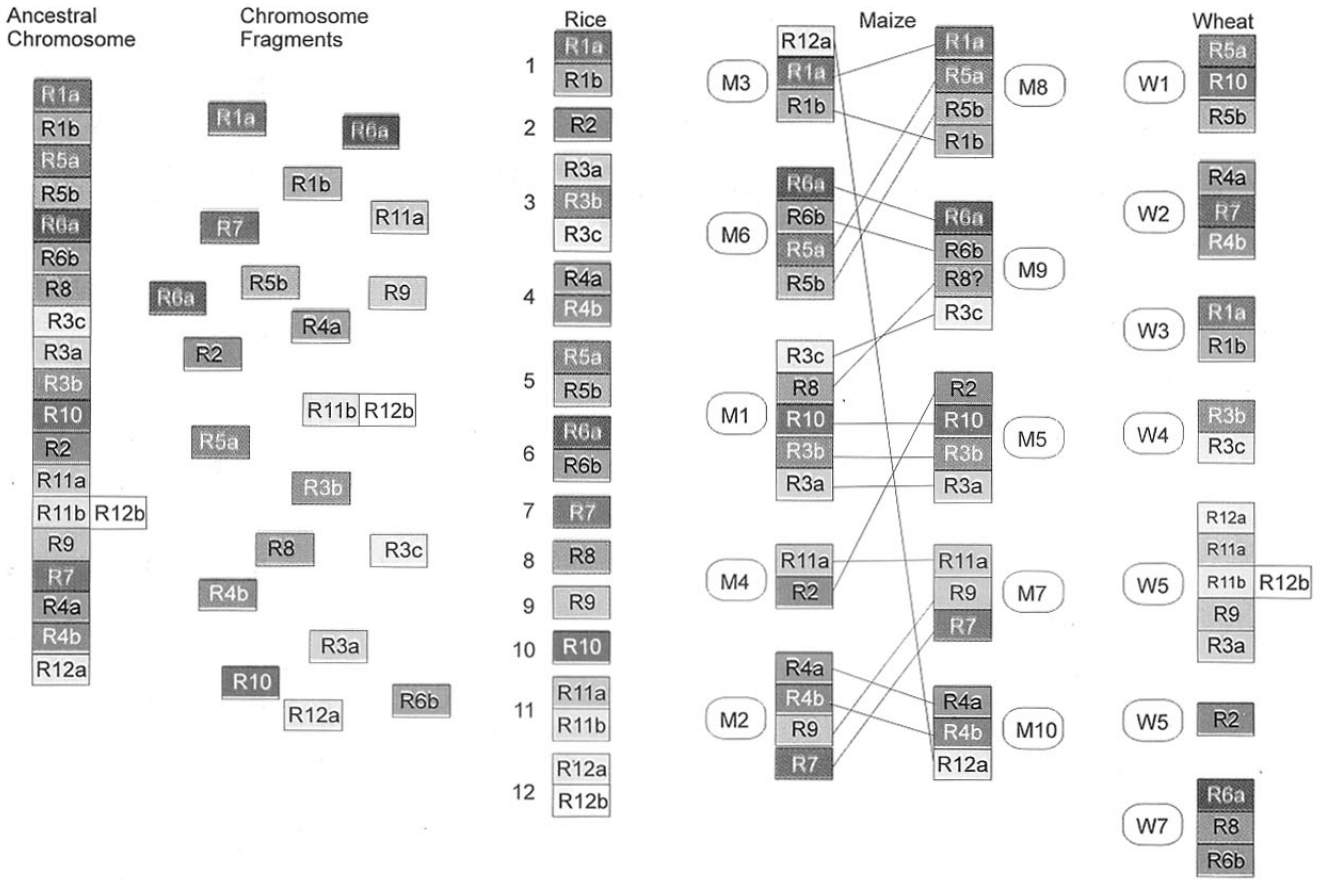



**Fig. 7.22.** Hypothesis of chromosome evolution in Gramineae family plants. Composition of ancestral 19 chromosome fragments in rice, maize and wheat chromosome complements is shown (modified from Moore et al., 1997).

the genome size. Actually, wheat has large blocks of heterochromatin around centromeres and gene spaces are reported to be localized on distal ends of chromosomes (Gill et al., 1996), whereas rice has chromosomes with many genes evenly distributed on them (Cheng et al., 2001). Larger genome size causes longer heterochromatic regions, which include very few genes. Therefore, the rice genome should have a gene space region similar to other cereals, but the smallest portion of repetitive sequences in most cereals.

Though the whole rice genome sequence is almost completed, little is known yet about its structural and organizational features. However, high density genetic maps including many gene/cDNA and microsatellite marker locations (Kurata et al., 1994; Causse et al., 1994; Harushima et al., 1998), have proven very useful for multiple purposes. One is a useful marker for selecting target genes in breeding programs. In the process of making many molecular genetic maps of different crosses, classical linkage groups composed of phenotypic markers were merged to the molecular map. Another use is as a framework for constructing physical maps, which provide detailed chromosome structure information. Moreover, genome-wide distribution of a large number of DNA markers has made it possible to detect long range duplication of chromosome segments in the distal part of short arms on Chromosomes 11 and 12 (Wu et al., 1998). Physical and genetic mapping of the two duplicated regions showed that these two regions had similar physical length and similar gene distribution, but revealed differences in genetic length. Both regions spanned at least 1.8 Mb, but the distal one-third was 0.3 cM in 11S (short arm of Chromosome 11) and 5.8 cM in 12S (short arm of Chromosome 12); the middle one-third was 3 to 4 cM in both, and the proximal one-third 5.1 cM in 11S but 0.3 cM in 12S (Fig. 7.21). This might indicate differences in chromosome function or organization in the duplicated regions. Results of full genome sequencing might shed light on these possibilities.

## Genome Synteny and Chromosome Evolution in the *Gramineae* family

One more useful and interesting result was derived from comparative mapping experiments among cereal genetic maps. In the middle 1990s, several other cereal molecular maps for maize, barley, and wheat were constructed and became available. Among cereal crops, there were many homologous genes that showed high similarity among them. Those genes were mapped on most different cereal maps as common markers. Comparative mapping using many common DNA markers along twelve rice chromosomes and other cereal chromosomes revealed a striking synteny among cereal crop chromosomes. Highly conserved genome structure for gene order between chromosomes of rice and other cereal species was thus detected among genomes of the Gramineae family (Moore et al., 1995a; Devos and Gale, 1997). Synteny was first realized between rice and maize (Ahn and Tanksley, 1993), between rice and wheat genetic maps (Kurata et al., 1994a), and was expanded to other cereal plants, e.g. barley, sorghum and millet, as well as sugarcane (Devos and Gale, 1997). Genome relationships between rice and maize showed an interesting result, namely that maize chromosomes are composed of doubled fragments of rice chromosomes with different combinations. Moore et al. (1995b) proposed a speculative ancestor chromosome for all cereal plant

chromosomes by reconstructing chromosome blocks with certain syntenies. Figure 7.22 represents an interesting hypothesis of chromosome evolution in family Gramineae indicated by Moore et al. (1995a, b) with many synteny experiments. The whole rice genome could be divided into 19 segments, which corresponded to either chromosome arms or chromosomes. Though all cereals have different chromosome numbers, each chromosome complement of rice, wheat, maize, foxtail millet, sugarcane, and sorghum were revealed to be composed of just 19 chromosome segments with different combinations. From this fundamental knowledge, one could postulate that all cereal chromosomes have one ancestral genome with 19 chromosomal segments. Discovery of synteny relationships among Gramineae species has provided an attractive strategy of using the rice genome as a standard model for Gramineae genome and chromosome analysis. The small rice genome size also makes it a model plant for monocot plant genome research.

High synteny was soon extended to gene isolation trials of homologous genes in cereals of a larger genome size, such as barley and wheat, by use of physical mapping of rice target regions (Killian et al., 1995; Foote et al., 1997; Chen et al., 1997). Results revealed that even on the physical map, gene order and distance are much the same among cereal genomes. This also indicates that gene space regions on chromosomes are similar in length among cereals; expansion of chromosome size was mainly achieved by amplifying repetitive sequences to form rare gene spaces outside the usual gene spaces.

## PROSPECTS FOR RICE CHROMOSOME RESEARCH AFTER GENOME SEQUENCING

### Chromosome Studies for Genome Differentiation Analysis

Sequencing along the entire length of 12 chromosomes well soon be accomplished in one Japonica rice strain of *O. sativa*. However, in addition to *O. sativa* (AA genome species), rice has another 24 wild and cultivated species in 9 different genomes of AA, BB, CC, BBCC, CCDD, EE, FF, GG, and HHJJ (Vaughan. 1994, and related papers in this book). After completion of genome sequencing in *O. sativa*, studies of genome diversity will continue to be an issue for genome analysis using the benefit of abundant species. These studies will necessarily include problems of genome organization and comparison at the chromosome level, sequence level, functional level, and evolutional level. There will be many mechanisms to organize structural and functional dynamics of the genome and studies for analyzing them will need some breakthroughs in molecular biological, cytogenetical, biochemical, or genetical methods. Molecular methods detecting structural differences at the nucleotide level as certain kind of polymorphism, such as RFLP (restriction fragment length polymorphism), AFLP (amplified fragment length polymorphism), CAPs (cleaved amplified polymorphisms), and SNPs (single nucleotide polymorphisms) are very powerful to illustrate genome differences between genetically related strains or species. On the contrary, quantitatively large differences present among species or genomes are difficult to be resolved for quantitative and qualitative difference location on chromosomes. At the level of gross differences, chromosome research in genome structure would yield important information, obtainable by no other biochemical or genetic method.

One example of relative genomic differences among B, C and D genomes was clearly shown by McGISH for intergenomic hybrid (see Figs. 7.14 and 7.16). Another way to see the extent of homology between homoeologous chromosomes in different genomes will be detection of chromosome pairing in the meiosis of $F_1$ hybrid. Basic homology between chromosomes should be observed in the pachytene stage where homologous and homoeologous chromosomes pair each other at the highest affinity. To date, however, pairing status of homologous chromosome was mostly observed in meiotic metaphase I some time after chromosome pairing event. Little was known about pairing status, region, duration, and strength in the pachytene stage of interspecific hybrids. If pairing feature details were analyzed in interspecific and intergenomic hybrids, not only the chromosome homology range necessary for pairing, but also chromosome organization in meiotic nuclei could be revealed. Molecular cytology might find a new aspect in the chromosome recognition process by using hybrid nuclei of meiosis.

As repetitive sequences, which are thought to participate in genome differentiation, become easier to detect, their distribution on chromosomes of different species will be ascertained. The extent of combination and distribution of those repetitive sequences will be detected on chromosomes in several more rice genome species, as examplified in Figure 7.10 and also Figure 7.15. Such techniques together with full sequencing results will help rice chromosome studies to reach a new standard.

## Chromosomes in Genome Organization Machinery

Genome sequencing can provide tremendous information about the primary structure of chromosomes as nucleotide arrays. Distribution of genes, repetitive sequences, and other specific sequences become clarified along the whole length of 12 chromosomes. Comparison of positions of characteristic sequences with chromosome bands in mitosis, with chromomeres in meiotic pachytene stage or only with chromosome physical positions, may reveal the structural context at each chromosome region. Recently Cheng et al. (2001) showed fine FISH mapping on pachytene Chromosome 10 with high resolution of physical distance. Such a technique could yield more information about the chromosome structure relation with genetic and biochemical functions.

Meanwhile, chromosomes are composed of many kinds of proteins and RNA, together with DNA. DNA, a core element of chromosomes, is often changed by modification through, for example, methylation. Their biochemical and genetic activities of chromosomal proteins are also changed by acetylation and phosphorylation. The dynamic nature of chromatin/chromosome structure and participation in chromosome function and nuclear organization should be an interesting topic in chromosome studies. Rice is estimated to have 50,000 genes, which is the largest number in all organisms sequenced to date and about half the number of *Arabidopsis*. How many genes are active under usual development and growth phases? How many genes are latent in the usual condition and await environmental changes for their activation? How is chromosome structure or nuclear architecture restricted to that biological status? There are no answers yet for how these gene functions accord with chromosome organization, or how chromosomes receive and react to various environmental stimuli. If nuclear and

chromosome organization phenomena were analyzed using rice chromosomes, several critical stages in development or at the changing point of growth conditions would undoubtedly prove beneficial for further analysis. Approaches for resolving these kinds of problems in plant chromosomes have rarely been applied. This would open attractive chromosome work as basic biology in plants. Chromosome studies will thus continue to offer various new strategies once rice genome sequencing has been completed.

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