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Localization of ribosomal RNA genes on rice chromosomes

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Abstract. Although it has long been known that the number of nucleoli in rice somatic cells at telophase varies from two to four, the number of nucleolar chromosomes has been determined only recently. This paper reports location of the rRNA genes by *in situ* hybridization with rDNA probes. The genes coding for 17S-5.8S-25S rRNA were located to the end of the short arms of chromosomes 8 and 10; those for 5S rRNA were located to the short arms of chromosome 7, 9 and 11, very close to the centromere. The number of the repeating units on chromosome 8 was found to exceed that on chromosome 10. It is also suggested that the number of nucleoli at pachytene, as well as at prometaphase, does not necessarily correspond to the number of nucleolar chromosomes, which is species (or subspecies) specific; this could arise by the fusion of the rRNA molecules transcribed from genetically similar rDNA alleles at different loci.

Key words: *In situ* hybridization; rDNA; Rice.

Introduction

In higher eukaryotes, the ribosomes contain four species of rRNAs. Three of them, 25-28S, 5.8S and 5S rRNAs, are associated with the 60S ribosomal subunit, and 17-18S rRNA is associated with the 40S subunit. Ribosomal RNA genes (rDNA) are tandem repeats located in the nucleolar organizer region(s) (NOR) of a genome (Appels and Honeycutt, 1986); each repeating unit contains the transcribed sequences coding for 17-18S, 5.8S and 25-28S rRNAs and the nontranscribed intergenic space region, the spacers (IGS). The 5S rRNA genes are generally not incorporated in such repeating units, but are located at other chromosomal sites. They may be clustered at one to three sites which are separated from the former gene clusters, as in wheat, barley, (Appels *et al.*, 1980; Reddy and Appels, 1989), maize (Mascia *et al.*, 1981), pea (Ellis *et al.*, 1988) and tomato (Lapitan *et al.*, 1991). Recent studies in restriction enzyme digestion of rDNA have revealed the phenomenon of dichotomy in most plant genomes. The sequences of their rRNA coding regions are highly conserved whereas the nontranscribed intergenic region shows extensive sequence divergence or size heterogeneity (Flavell, 1986; Rogers and Bendich, 1987; Hemleben *et al.*, 1988; Reeder, 1984).

Most of the published papers indicate that rice rDNA is heterogeneous (Oono and Sugiura, 1980; Olmedilla *et al.*, 1984; Pental and Barnes, 1985), though a single type of repeating unit has been reported in cultivar IR20 (Reddy and Padayatty, 1987). The coding sequence of a cloned rice rDNA unit was determined by Takaiwa *et al.* (1984, 1985a, 1985b), so that detailed information on this part of the gene is available and has been used to investigate the variation of rDNA intergenic spacer in the genus *Oryza*. According to studies of restriction enzyme map, variation in the copy number of small subrepeats accounts for the length heterogeneity of the rDNA intergenic spacer. The subrepeats may vary considerably in length or in copy number from one species to another (Cordesse *et al.*, 1990; Sano and Sano, 1990). Similar variation or modification in the coding sequence, as well as in the

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intergenic spacer, of plant 5S rRNA genes has also been reported recently (Erdmann *et al.*, 1984; Erdmann and Wolters, 1986; Vanderberghe *et al.*, 1984; Barciszewska *et al.*, 1985; Hariharan *et al.*, 1987).

From cytogenetical studies, the number of nucleolus in rice root tip cells has been reported to vary from two to four among cultivars, including indica and japonica types, and even among species (Selim, 1930; Oka and Kao, 1956; Shinohara, 1962). However, no information about variation in the number of nucleolar chromosomes has been available until recently. Through cytological investigations, many workers indicated that chromosome 10 is the only nucleolar chromosome in most rice species, including those which are cultivated (Kuruta and Omura, 1978; Kurata *et al.*, 1981; Chen *et al.*, 1982; Chen and Wu, 1982); but chromosome 8 was designated the nucleolar chromosome in *Oryza australiensis* (Chen and Wu, 1982). Rice clultivar IR36 (Chung and Wu, 1987), one of the most popular indica type cultivars, as well as a wild rice species, *Oryza perennis* (Chen and Wu, 1982), have two pairs of nucleolar chromosome, chromosome 8 and 10, at both the meiotic pachytene and the mitotic prometaphase stages. Even though these observations parallel the variation in the number of nucleoli, there is no direct evidence to show that the morphologically identified nucleolar chromosomes do, in fact, carry rRNA genes. In this study we have located, by *in situ* hybridization, the rDNA coding for the 17S-5.8S-25S rRNA at the end of the short arm of chromosomes 8 and 10, and that for 5S rRNA in the short arm regions, very close to the centromere of chromosomes 7, 9 and 11.

Materials and Methods

DNA Probes

Two clones of rice rDNA sequences, pRY12 and pRY18, were kindly provided by Dr. Sano. The DNA segments coding for rice 17S-5.8S-25S ribosomal RNA had been cloned recently by Sano (1988), before studies on the intergenic space region of rice rDNA (Sano and Sano, 1990). rDNA repeats were subcloned from EMBL 4 genomic clones into the BamHI site of pUC13. The length of a rDNA repeating unit isolated from Taichung 65 (*Oryza sativa*, japonica type), is 8.3kb. BamHI cuts each repeating unit into two fragments, one carrying the intergenic spacer region (pRY12) and the other containing the major coding sequences (pRY18), the lengths of which are 4.5kb and 3.8kb respectively (Fig. 1).

Another clone, pRTY5S, containing rice 5S rDNA sequence was constructed and kindly given by Prof. Ray Wu of Cornell University, USA. An approximate 3kb insert, containing a 5S rDNA repeating sequences, was cloned in the pTZ19R vector at EcoRI sites, and transformed into *E. coli* strain JM101.

In situ Hybridization

The preparation of rice prometaphase chromosomes, H^3-labelling of DNA probes and *in situ* DNA hybridization to rice chromosomes were carried out as described by Wu *et al.*, 1991. In this study, *Oryza sativa* cultivars IR36 (indica type), Taichung 65 (TC 65, japonica types) and Chianung 242 (CN 242, japonica types) were used. After denaturation, the chromosomal DNA

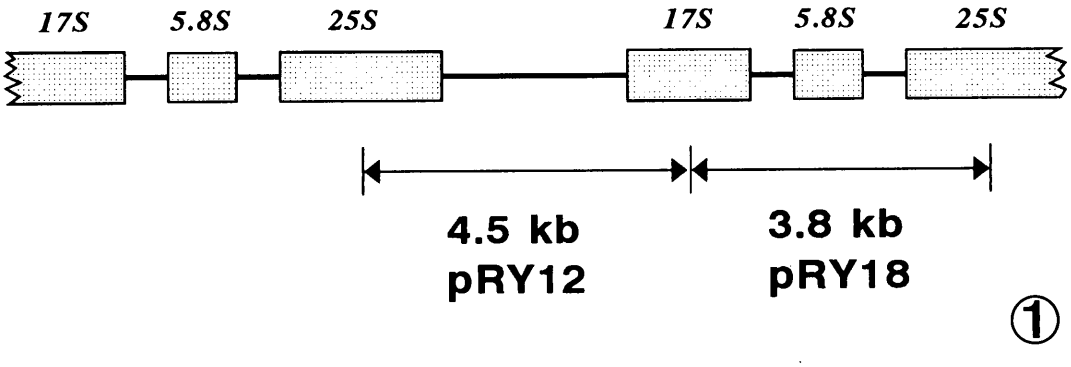


Fig. 1. The figure depicts the size and extent of the two probes, pRY12 and pRY18, prepared from the repeating unit of rice rRNA genes sequences.

Table 1. *Relative lengths and arm ratios of IR36, Taichung 65 and Chianung 242 chromosomes at prometaphase*

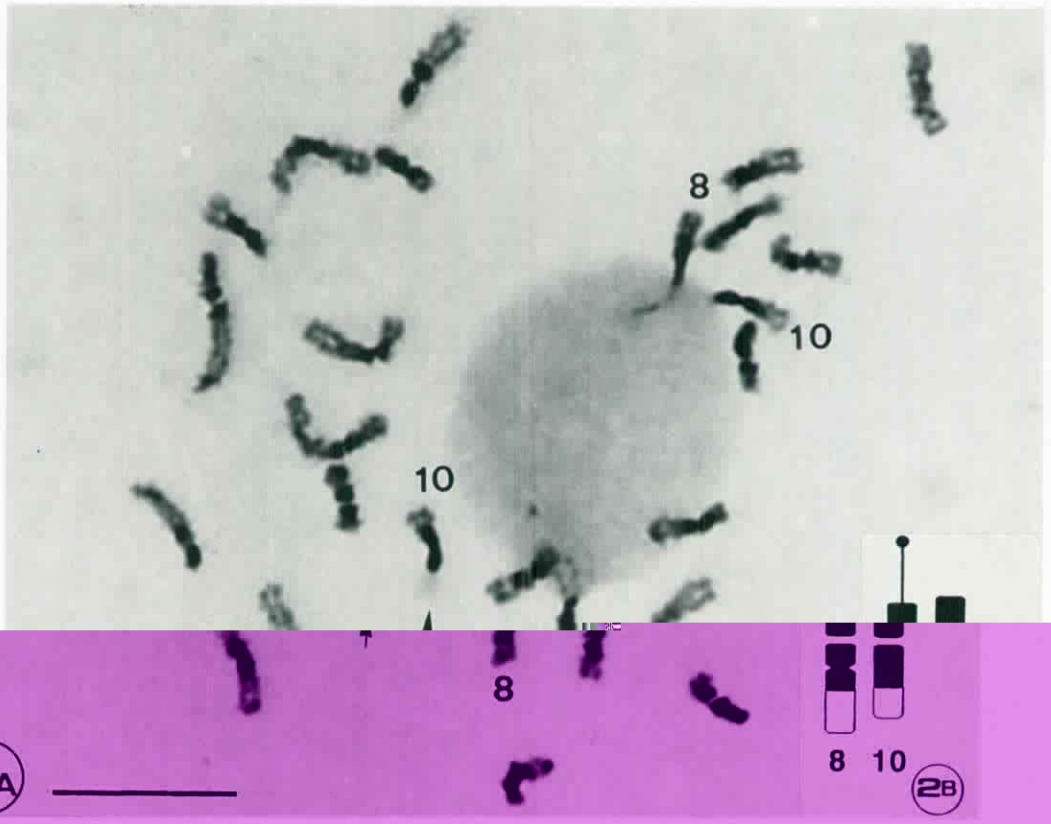
Chromosome	IR36 (35 cells) RL	L/S	Taichung 65 (30 cells) RL	L/S	Chianung 242 (19 cells) RL	L/S
1	13.81	1.81	12.82	1.94	14.18	1.88
2	11.70	1.28	11.46	1.41	11.96	1.29
3	11.12	1.88	10.42	2.00	10.74	1.63
4	9.16	3.72	9.20	3.99	9.03	3.63
5	8.71	1.78	8.32	1.92	8.21	1.87
6	7.91	1.21	8.00	1.26	8.25	1.20
7	7.26	1.95	7.30	1.81	7.09	1.76
8	6.69[n]	3.05	7.09[n]	4.14	6.17[n]	3.34
9	6.51	1.39	6.95	1.41	6.75	1.29
10	6.02[n]	2.88	5.69	2.52	5.85	2.16
11	5.79	1.79	6.40	1.48	6.10	1.46
12	5.33	1.38	6.00	1.34	5.50	1.29

RL: Relative length, length as percentage of the total length.
L/S: Arm ratio, long arm /short arm.
n: Nucleolar chromosome.

was hybridized *in situ* with each of the H^3-labelled DNA probes. The specific activities of these probes were approximately 4×10^7 cpm per microgram DNA. For coating the hybridized slides, Amersham photographic emulsion LM-1 was diluted with 0.5% gelatin in 1:1 ratio. The coated slides were exposed in a dry, light-proof box at 4°C for seven days. Slides were developed in Kodak D19 (diluted two-fold with water), fixed in Kodak Fixer at 15°C, stained with 4% Giemsa in 1/15M phosphate buffer and then differentiated by rinsing with tap water for a few seconds. The chromosome numbering system used was that described by Chung

mosome 8, a discernible thread-like secondary constriction and a small dotted satellite extending over the nucleolus were seen in most of the observed cells (Fig. 2, insert). In general, both chromosome pairs, 8 and 10, were usually associated with one large nucleolus; but in some cases at prometaphase, one of chromosome 10 was connected to a smaller nucleolus beyond the large one (Fig. 2).

Japonica cultivars, TC65 and CN242, have only one pair of chromosomes associated with the nucleolus. They have been designated as chromosome pair 8, by the relative length, arm ratio and morphological simi-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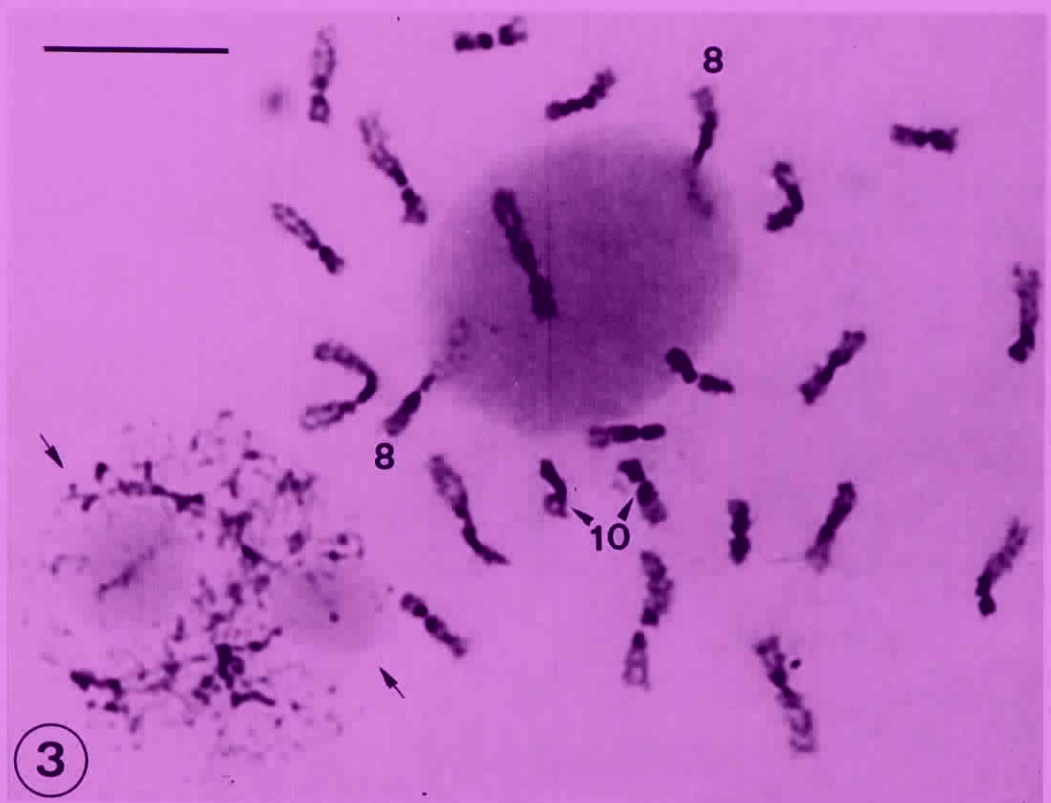
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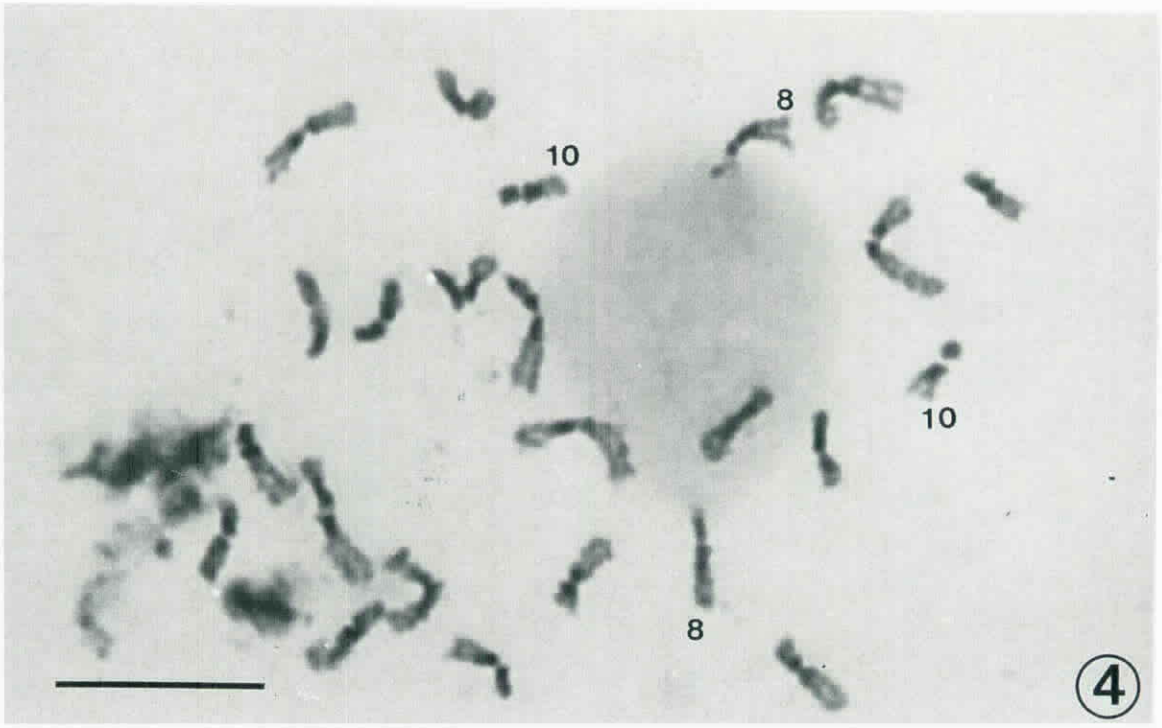
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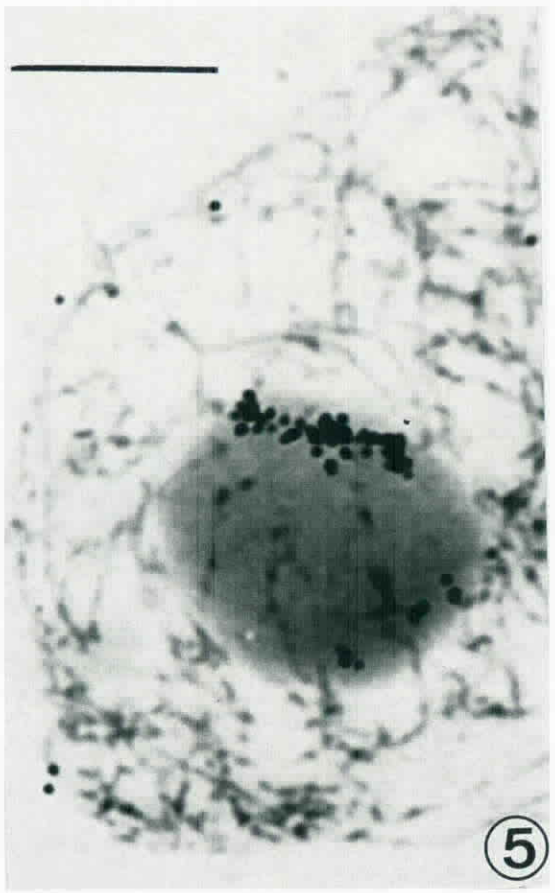
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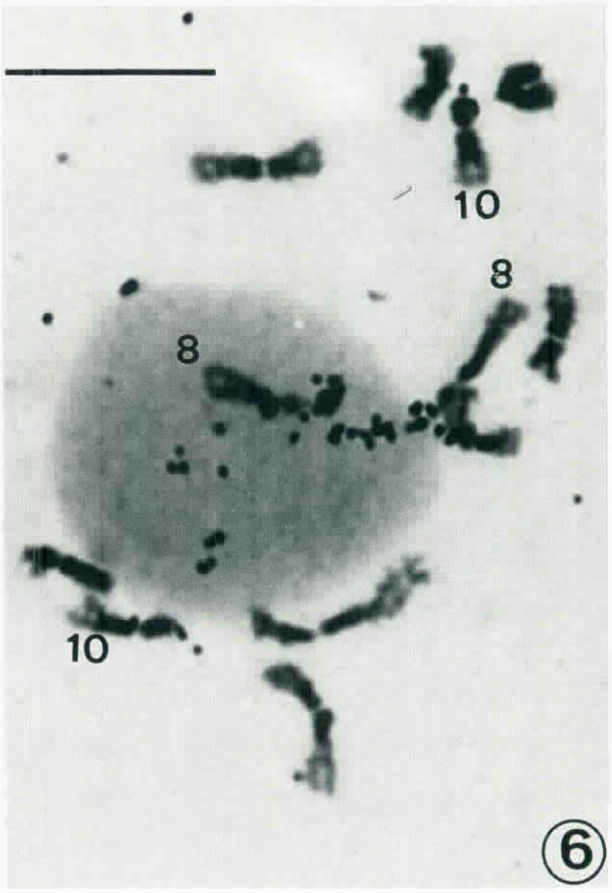
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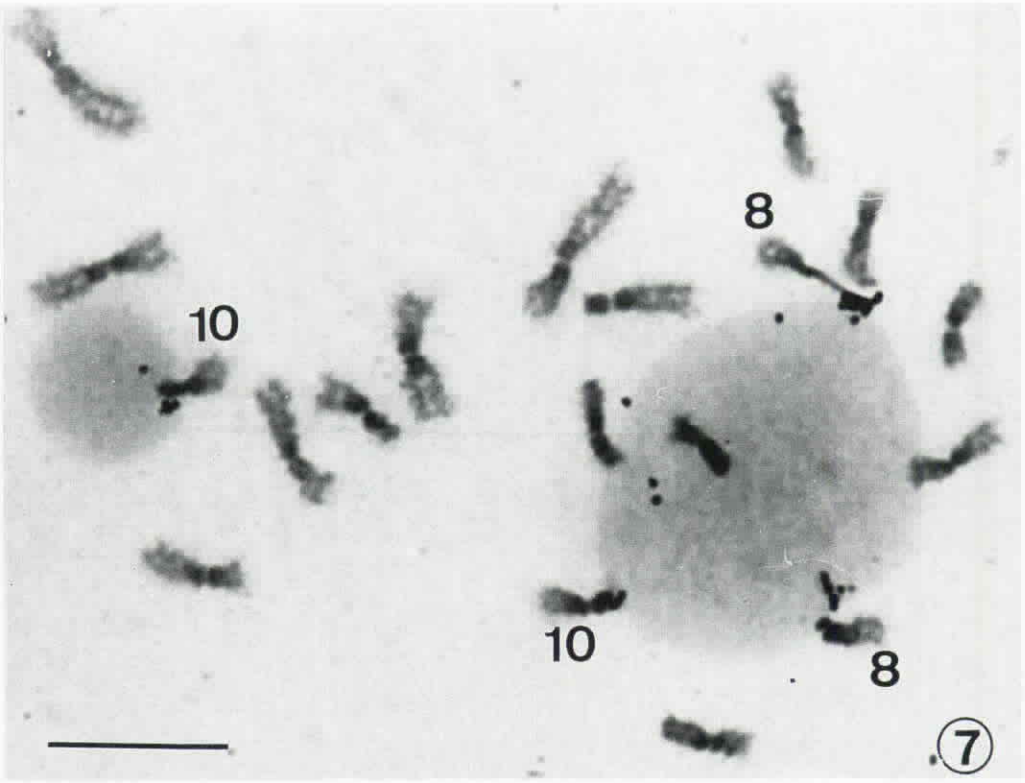
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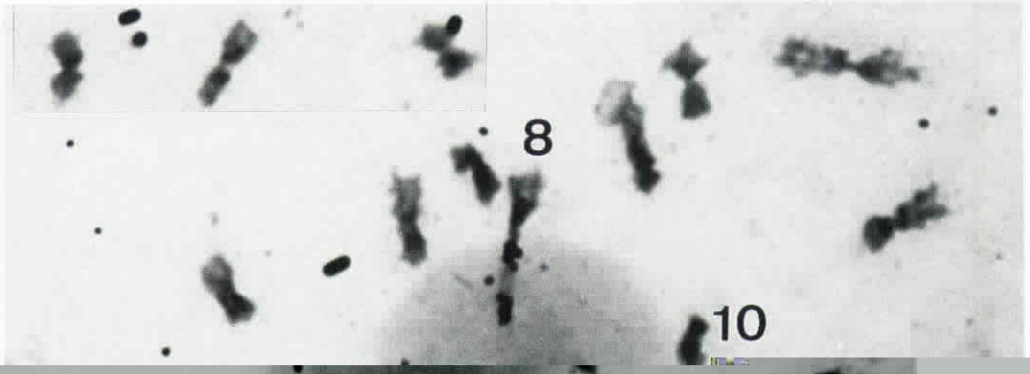
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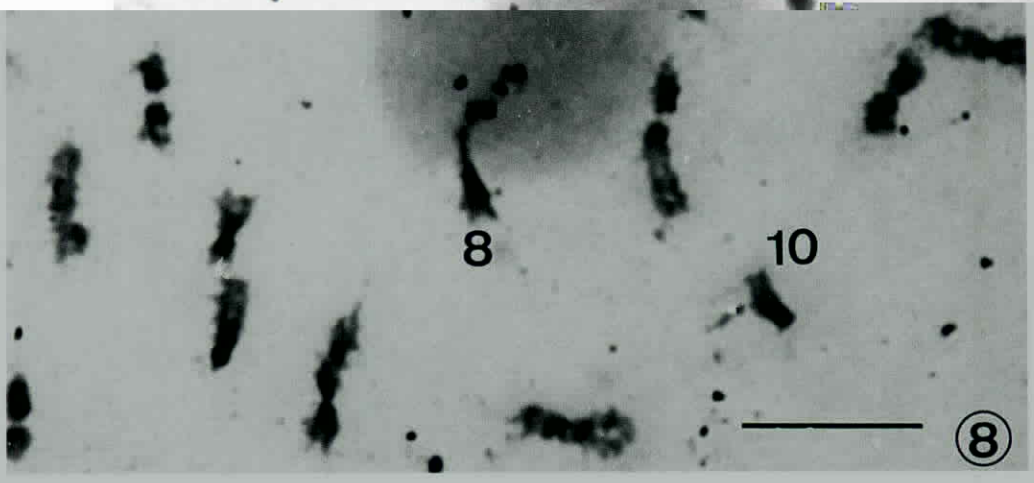
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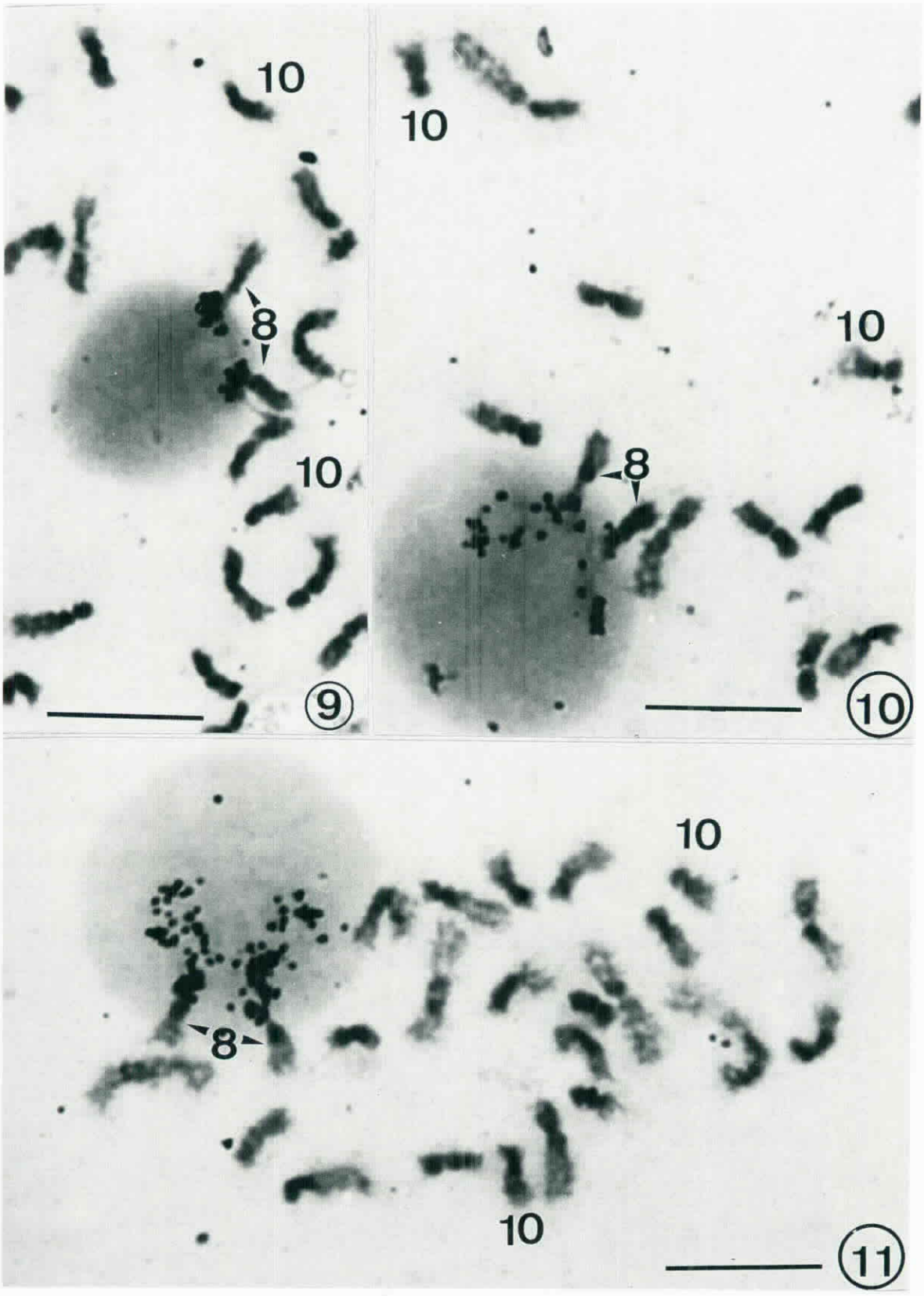
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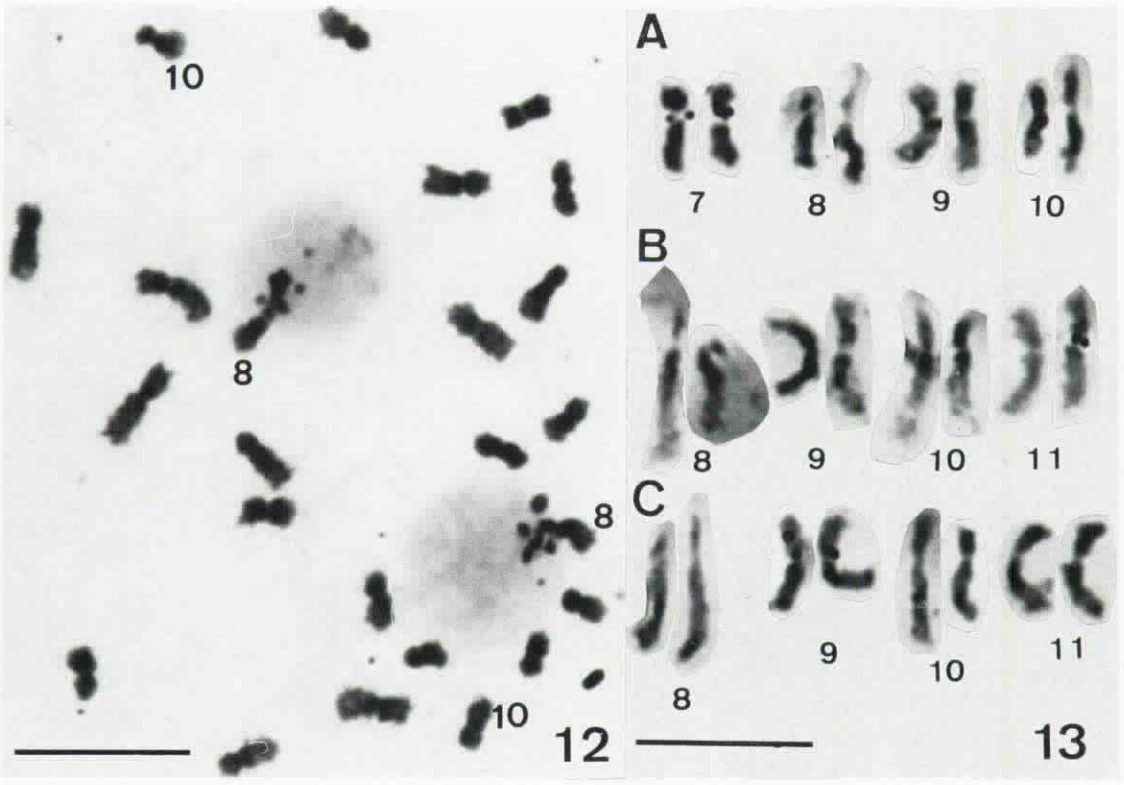

Figs. 2–4. The chromosome complement of rice cultivars. Note that nucleolar chromosomes are associated with the nucleolus at prometaphase. (Scale = 10 μm). Fig. 2. A shows that in IR36 two chromosomes 8 and one chromosome 10 are associated with the large nucleolus; another chromosome 10 has its own small nucleolus (indicated by an arrow). The attachment is confined to the short arm end of chromosome 10, but the thread-like secondary constriction and the dotted satellite extending from the short arm end of chromosome 8 are seen to be involved in the association; Fig. 2B. Nucleolar chromosome pairs 8 and 10 are distinguishable from each other by the criteria that the former is longer, has larger arm ratio and by the thread-like secondary constriction and the dotted satellite; Fig. 3. In TC65, only two chromosomes 8 are attached to the nucleolus with their thread-like secondary constriction and dotted satellite. In a cell at prophase, two nucleoli (each indicated by an arrow) are shown; Fig. 4. In CN242, only two chromosomes 8, having their morphology similar to those of IR36, are found attached to the nucleolus.

Figs. 5–7. Silver grain distribution in IR36 after in situ hybridizing the chromosome complement with two probes. Fig. 5. At prophase, silver grains are concentrated at part of the IR36 nucleolus where the nucleolar chromosomes are attached. Fig. 6. This shows an exception in IR36. Only two chromosomes 8, that have been heavily labelled using pRY12, are attached to the nucleolus; minor labelling can be found also on the free chromosome pair 10 at their short arm ends. Fig. 7. In IR36, two nucleoli, one large and one small, can often be found. Using pRY18, two chromosomes 8 attached to the large nucleolus are labelled with more silver grains than are found on the two chromosomes 10 which are attached respectively to the two nucleoli. (Scale = 10 μm)

Figs. 8, 9, 12. Silver grain distribution in TC65 after in situ hybridizing the chromosome complement with two probes. The thread-like segment and the short arm end of chromosomes 8 are heavily labelled, respectively, using pRY12 (Fig. 8) and pRY18 (Fig. 9). In a cell with two nucleoli of almost equal size (Fig. 12) only chromosomes 8, each attached to one nucleolus, have their short arm ends labelled. (Scale = 10 μm)

Figs. 10–11. Silver grain distribution in CN242 after in situ hybridizing the chromosome complement with two probes using either pRY12 (Fig. 10) or pRY18 (Fig. 11). The grains are heaving distributed along the thread-like secondary constriction and the dotted satellite. (Scale = 10 μm)

Fig. 13. A, B and C. Labelling of the IR36 chromosome complement using pRTY5S (5S rDNA) as a probe. Silver grains can be detected on a region of the short arm, very close to the centromere on chromosome pairs 7 (Fig. 13A), 11 (Fig. 13B) and 9 (Fig. 13C). (Scale = 10 μm)

Table 2. *The variation of labelling strength of the rice rDNA clones, pRY12 and pRY18 as the probes by* ***in situ*** *hybridization on the nucleolar chromosomes of IR36, Taichung 65 and Chianung 242.*

	pRY12	pRY18
IR36		
$X_8 \pm SE$	9.07±10.08	10.14± 8.86
$X_{10} \pm SE$	3.43± 4.33	3.66± 3.91
cells analyzed(n)	46	25
t-value	9.5033**	8.9204**
$\Sigma(X_{8j}/X_{10j})/n$	3.23± 2.18	4.26± 4.29
Taichung 65		
X_8	10.92± 9.2	19.28±11.32
cells analyzed(n)	12	3
Chianung 242		
X_8	16.00±12.65	28.13±15.34
cells analyzed(n)	9	8

**The difference of labelling strength on chromosomes 8 and 10 are highly significant.

heavily labelled in TC65 (Figs. 8, 9) and CN242 (Figs. 10, 11).

The labelling strength of the two probes on the nucleolar chromosomes of IR36 was enumerated in Table 2. With pRY12 as a probe, in 46 IR36 prometaphase cells there was an average of 9.07 silver grains on the short arm end of chromosome 8 and 3.43 silver grains on that of chromosome 10. The average ratio of silver grains on chromosome 8 to those on chromosome 10 was 3.23. This means that the labelling on chromosome 8 is about three times stronger than that on chro-

number of pairs (n). The null hypothesis test was that the mean of the population of difference is zero; the alternative was that the mean is not zero. On the basis of the *t* values from both experiments, the null hypothesis were rejected. This means that the differences of labelling strength on chromosome 8 and 10 are significant in both hybridization experiments (Table 2). However, on either nucleolar chromosomes there is no significant difference of labelling strength between the two probes.

In IR36 cells at prophase, the distribution of most silver grains was apparently along the chromosome segments that associated intimately with nucleolus (Fig. 5). At prometaphase, the distribution along the secondary constrictions extending from the short arm ends of chromosome pair 8 (Fig. 6). The appearance of the labelled secondary constriction at prometaphase was even finer than that of chromosomes at pachytene. Morphologically chromosome 8 at pachytene stage also has a secondary constriction-like segment lying on the nucleolus (Chung and Wu, 1987). Our interpretation is that these thread-like secondary constrictions are chromatin fibers which extend from the condensed prometaphase regions of rRNA genes.

Furthermore, in some IR36 cells, there were two separated nucleoli, one large and one small. The small nucleolus was associated with labelled chromosome 10, while the large one was attached to the other three labelled chromosomes (Fig. 7).

In cultivars TC65 and CN242 (Figs. 8-11, inclusive) both probes labelled the ends of the chromosome 8

phase chromosome of rice is shown in Figs. 13 and 14. In one of our experiments, 35 cells of IR36 were analyzed. The number of silver grains distributed within those analyzed cells was 186, 62 of which were non-specifically labelled. The number of specifically labelled silver grains was 70 (37.63%), 38 (20.42%) and 16 (8.60%) on the short arm region close to the centromeres of chromosome 7, 11 and 9 respectively. It is very likely that the 5S rRNA gene cluster in rice is located mainly on chromosomes 7, 11 and probably 9

and that their distal ends are connected to one fused nucleolus. The connection is rather unique; each nucleolar chromosome pair has its own connection site, and the sites (two at pachytene and four at prometaphase) are usually close to each other and symmetric. In this study, we have confirmed these observations. Furthermore, nucleolar chromosome 8 has been shown to have a thread-like secondary constriction and a dotted satellite at prometaphase (Figs. 2, 3, 10 and 11). In

difficult to do further analysis by southern blot. Pulsed field eletrophoresis would provide an alternative way through separation of large DNA segments, each with one of the two rDNA loci; their heterogeneity could then be determined separately.

In addition to heterogeneity in length of the subrepeating unit in IGS among rice cultivars (Sano and Sano, 1988) or species (Sano and Sano, 1990; Cordesse *et al.*, 1990), there may be also heterogeneity in base alternation, though it has not yet been confirmed. In our observations, we noted that most IR36 cells at prometaphase had a large nucleolus associated with one pair of chromosome 8 and another pair of chromosome 10, but in some cells there was a large nucleolus plus a small one to which only chromosome 10 of the pair was attached. It is suggested that the expression level of the two rDNA loci in IR36 may be different from each other owing to the possibly altered base in methylation sites. It is known that active loci have a higher proportion of rRNA genes with unmethylated cytosine (Flavell *et al.*, 1988). In wheat, it has been shown that the expression level of rDNA locus at chromosome 6B is higher than that at all others, i.e. 6B > 1B >5D >1A (Cermeno *et al.*, 1984). Furthermore, methylated repeats and unmethylated repeats are interspersed, one with another, in the rDNA of radish and pea (Ellis *et al.*, 1988).

Japonica cultivars, such as TC65 and CN242, which are believed to have derived from indica cultivars, may have had their rDNA locus on chromosome 10 somehow deleted during breeding. If the rDNA locus were there but highly methylated, it should still have been labelled. In a mutant wheat with the IBS-2 locus deleted, no signal was detected during its *in situ* hybridization to 18S-26S rDNA clones (Mukai *et al.*, 1991.)

The nucleolus is the site for rRNA accumulation before release from nucleus to cytoplasm. Rice nucleoli can always be detected at pachytene by aceto-carmine staining (Wu, 1967) and at prometaphase by Giemsa (Kurata and Omura, 1978). However, it is not clear why there are two associated nucleoli at pachytene, but usually only one at prometaphase. We suggest that nucleolar fusion may occccur because the transcribed rRNA molecules are very similar to each other. In japonica cultivar, the size of those two components of a nucleolus are different which may result from the rDNA loci on chromosome pair 8 being heterozygous, or with different level of expression or number of repeating unit; otherwise, the two components would be of equal size. Beyond fusion, mixing may further occur at prometaphase, and a cultivar with a homozygous rDNA locus would then have one large nucleolus with one pair of nucleolar chromosome associated; this is what we have usually observed. In one of our experiments, however, we found a cell of TC65 with two separate nucleoli of equal size, each attached with one chromosome 8 (Fig. 12). This may support our view that the rDNA locus at nucleolar chromosome 8 in TC65 is homozygous and that similar amounts of rRNA have been transcribed, but the fusion and/or mixing of the nucleoli has not taken place. The suggestion may applied to the indica cultivars, where the rRNAs transcribed by two pairs of nucleolar chromosomes are usually fused to form a nucleolus with one large and one very small at pachytene as we showed in our previous paper (Chung and Wu, 1987). At prometaphase most IR36 cells have only one large nucleolus associated with two chromosomes 8 and loosely with two chromosomes 10 (Fig. 6). Alternatively, there may be a separated small nucleolus associated with one chromosome 10 beyond the large nucleolus associated with one chromosome 10 and two chromosomes 8 (Figs. 2 and 7).

More detailed studies of rRNA gene regulation sequences has not been carried out in plants but has been investigated in *Xenopus laevis*; in that species, three promoters and three transcription termination sequence elements have been identified in the IGS (Meissner *et al.*, 1991). They further found that transcription consecutively passed the termination sites (T1 and T2), resulting in read-through transcription of the entire rDNA spacer sequence up to transcription site T3 located immediately upstream of the 5' 40S main promoter element. Regulation of rRNA gene expression has yet to be studied in rice. However, the extending of the thread-like secondary constriction of the nucleolar chromosome 8 onto the nucleolus at prometaphase (Figs. 2 and 3) and the labelling pattern shown in figures 10 and 11 suggests that the transcription of rRNA at the secondary constriction of chromosome 8 is late. It is known that transcription of rRNA in human spermatocytes takes place at prophase i.e. from leptotene to zygotene (Stahl *et al.*, 1991).

The repeating unit in the 5S rRNA tandem array in plants is usually composed of only 300 to 400 base

pairs including the intergenic spacer with micro heterogeneous sequence; this has been shown in wheat (Gerlach and Dyer, 1980), maize (Mascis *et al.*, 1981), rye (Reddy and Appels, 1989), soybean (Gottlob-McHugh *et al.*, 1990) and tomato (Lapitan *et al.*, 1991). Only one 5S rDNA locus was found in tomato and maize but there are two in rye. Rice has a 5S rDNA array structure very similar to that of wheat, rye and maize (Hariharan *et al.*, 1987) and we have, in this paper, localized the array to chromosome pairs 7, 11 and 9.

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稻核糖體 RNA 基因在染色體上的定位

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十年前已知稻體細胞分裂末期通常有 2-4 個核仁，核仁染色體數目的確定則爲近年之事。本文以稻核糖體 RNA 基因 (17S-5.8S-25S 及 5S rRNA) 的片段爲探針與稻體細胞早中期染色體作原位雜交，確定此等基因位於稻第八及第十兩對染色體 (即核仁染色體) 短臂末端。5S rRNA 則位於第七、十一及九各對染色體短臂近中節處。第八對染色體上的 rDNA 的重複單位片段數目遠超過其在第十對染色體上的。稻減數分裂粗絲期及體細胞分裂早中期細胞內的核仁數目不一定與其核仁染色體數目相當，此乃由於從對偶 rDNA (在第八對及/或第十對染色體) 轉錄的 rRNA 分子有相當的相似性，因而相互融合所致。第八對染色體短臂末端線狀次元次縊痕於早中期，似仍有部分轉錄在進行。