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The physical location of fourteen RFLP markers in rice (*Oryza sativa* L.)

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Abstract A biotin-labeled in situ hybridization technique was used in order to physically map RFLP markers to the chromosomes of rice (*Oryza sativa* L.). Fourteen RFLP markers, associated with the ends of the linkage groups on rice chromosomes 7, 8, 11, 12, were physically mapped onto specific regions of the chromosomes. The average detection rate of in situ hybridization was 5.91%. The markers were located on seven different chromosome arms. Ten of the fourteen markers were distributed near the chromosome ends. This demonstrated that the RFLP linkage groups involved covered a wide physical distance and that the centromeric region was bisected by all but one linkage group. Two markers covered a short genetic distance but were physically distant, while two covering a longer genetic distance were physically closer together. This indicates that considerable variation can, and does, exist between genetic and physical maps.

Key words In situ hybridization · Biotin labeling · DNA sequence mapping

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Introduction

RFLP linkage maps have been developed in rice (*Oryza sativa* L.). McCouch et al. (1988) mapped 135 RFLP genomic clones and gene markers, Tanksley et al. (1989) mapped 144 RFLP genomic clones and gene markers, and Saito et al. (1991) mapped 350 RFLP genomic clones. The number of mapped RFLP genomic clones is thus steadily increasing. These RFLP linkage maps have a number of advantages over classical genetic maps and offer new opportunities for applications in genetics and breeding. However, at present, the RFLP maps and physical maps often show that, while markers have the same order along the chromosome, there may be little correlation between the separation distances of markers on the two types of map. Gustafson and Dillé (1992) reported that there was considerably variation between the genetic and physical maps of rice. Genetic linkage, and gene and sequence interdependence, can be understood only by developing the physical maps (Heslop-Harrison 1991).

Within the genus *Triticum* it has been noted that the physical distances between genes are often quite different from the observed genetic distances (Dvorak et al. 1984; Sears 1984; Snape et al. 1985; Dvorak and Appels 1986; Curtis and Lukaszewski 1991; Lukaszewski and Curtis 1993). It seems that the distal part of cereal chromosomes show a significantly-higher degree of genetic recombination than does the chromosome region proximal to the centromere (Linde-Laursen 1982; Jampates and Dvorak 1986; Curtis and Lukaszewski 1991; Lukaszewski 1992), a situation which has been observed in other plant and animal species. Lukaszewski and Curtis (1993) go on to indicate that there can be a recombination distribution difference of 153-fold in the number of basepairs (bp) per cM (centimorgan). However, their statistics assumed that the DNA was equal in density along the length of the chromosome, and did not take into account the presence of heterochromatic blocks or regions with different chromosome coiling and

condensation which may influence recombination. It appears that in wheat, *Triticum aestivum* L. em Thell., the frequency of recombination dramatically increases the further from the centromere that loci are located, as was noted when analyzing telocentric chromosomes (Sears 1972).

It has even been observed that there can be a difference in the distribution of recombination frequencies between short and long chromosome arms of various cereals (Gustafson and Dillé 1992; Lukaszewski and Curtis 1993). Gustafson and Dillé (1992) went on to show that significant differences between physical and genetic differences can also occur within an arm of a chromosome. In rice there did not appear to be any pattern to the recombination differences, and variation between the physical and genetic distances could occur on either the short or long arms.

At the present time, in situ hybridization (ISH), or variations on this technique, appear to be the best method for the physical mapping of genes or RFLPs on chromosomes. This technique can be used to physically map all classes of DNA sequences directly to the chromosomes of any plant or animal species. Up to now, using ISH, both repeated DNA sequences (Appels et al. 1980; Hutchinson et al. 1981; Mascia et al. 1981; Appels and McIntyre 1985; Mukai et al. 1990; Leitch et al. 1991; Maluszynska and Heslop-Harrison 1991; Moore et al. 1991; Mukai et al. 1991) and low-copy or unique sequences (Ambros et al. 1986; Mouras et al. 1987; Shen et al. 1987; Huang et al. 1988; Simpson et al. 1988; Clark et al. 1989; Shen and Wu 1989; Gustafson et al. 1990a, b; Gustafson and Dillé 1992) have been mapped in some plant species. The above studies have provided novel information about genome organization, gene activity, recombination, and the application of the physical mapping of genes and DNA sequences to plant breeding. The results of ISH in rice (Gustafson and Dillé 1992) indicated that the linkage maps did not cover the entire genome and that there were several non-polymorphic regions on different chromosomes. Linde-Laursen (personal communication) confirmed the presence of active and inactive gene loci and physically mapped their sites on chromosomes in *Hordeum marinum* ssp. *gussoneanum* (4x). Moore et al. (1991), using ISH, found that an element of the genome, which comprised some 5% of the total DNA of barley (*Hordeum vulgare* L.), was located over most chromosome arms, except for the telomeric and nucleolar-organizing regions.

The chromosomes in rice are very small and consequently ISH is more difficult in rice than in many other plants. Combining improvements to the protoplast technique, developed by Dillé et al. (1990) for making high-quality chromosome preparations, with biotin labeling with enzyme-conjugated reporters (Ambros et al. 1986; Simpson et al. 1988; Gustafson et al. 1990a,b; Gustafson and Dillé 1992) for reducing nonspecific hybridization signals and for improving the detection of target sequences, has allowed for the mapping of RFLP clones to rice chromosomes. Twenty-three rice RFLP markers and the 5*S* DNA gene complex located on chromosomes 1–6, 9–10 have been physically mapped (Gustafson and Dillé 1992; Song and Gustafson 1993). The present study was designed to physically locate the remaining RFLP linkage groups from the Tanksley et al. (1989) map to chromosomes 7–8, 11–12 and to analyze the relationship between the genetic and physical maps. The new rice chromosome numbering system, adopted at the 2nd International Rice Genetics Symposium, Manila, The Philippines, was utilized in the present study. This new system changed the numbering of chromosome 6 to its present number, chromosome 12.

Materials and methods

Materials

Plants. *O. sativa* cv 'IR 36' was supplied by Dr. G. Khush, International Rice Research Institute, Los Banos, The Philippines.
DNA probes. The RFLP probes, which include RG29, RG4, RG170, RG165, RG30, RG20, RG28, RG365, RG353, RG118, RG181, RG361, RG98, and RG190, were supplied by Dr. S. R. McCouch, Department of Plant Breeding and Biometry, Cornell University, Ithaca, New York, USA. All clones are present in the plasmid pUC8.

Methods

Chromosome preparations. The protoplast technique employed was that developed by Dillé et al. (1990) with the following modifications. The harvested root tips were immediately fixed in ethanol-acetic acid (3:1) without pretreatment. After a 5 M HCl treatment for 90 min, the materials were digested in 1% cellulase-1% pectolyase (Karlan), for 50–60 min. The protoplast spreads were then prepared by the following steps: (1) the enzyme solution was removed from the root tips and 5–10 ml of deionized water was added; (2) a Pasteur pipette was used to carefully remove four root-tips and gently place them on a slide followed by blotting away the excess water; (3) a scalpel was used to break apart the root tips, thus making a protoplast slurry on the slide; (4) immediately, 2–3 drops of 3:1 fixative were added to the slurry; and (5) the slide was then tilted and rotated to spread the slurry uniformly.

The techniques of biotin labeling, in situ hybridization, and for the detection of hybridized probes were those used by Gustafson and Dillé (1992).

The in situ hybridization slides were analyzed using a Zeiss photomicroscope III and a Hamamatsu Image Enhancement system attached to a high-resolution television monitor. All chromosome measurements were made directly from the television screen with a pair of calipers. An average of the hybridization-site measurements was taken by calculating the distance from the centromere to the detection site and using that as a percentage of the arm on which the site was located. A percentage was used because it eliminated the problems associated with variation in chromosome condensation. The standard deviation was then calculated for the group of measurements. The arm ratio of the chromosome showing a detection site was also measured and standard deviations were calculated in order to determine if the site was located on the same chromosome. This was also a check for the potential occurrence of any spurious hybridization sites. The standard errors were so small as to minimize the possibility of having included any false hybridization sites in the measurements (see Table 1).

Results and discussion

Gustafson and Dillé (1992) physically mapped the end markers of the linkage groups (Tanksley et al. 1989) associated with rice chromosomes 1–6, 9–10. The

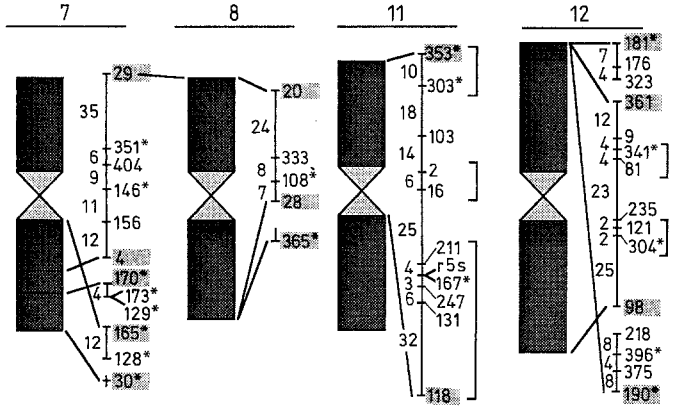


Fig. 1 The RFLP linkage map (Tanksley et al. 1989) and chromosome karyotypes showing the physical location of the hybridized RFLP markers for chromosomes 7–8, 11–12 in rice (*Oryza sativa* L.)

present study physically mapped 14 markers of linkage groups associated with chromosomes 7–8, 11–12 (Fig. 1). A total of 2082 late-prophase or early-metaphase spreads were analyzed and 123 hybridization sites (Table 1) were detected (5.91%). This rate of detection is very close to that (6.06%) reported by Gustafson and Dillé (1992). The results showed that the genomic clones RG30, and RG29 and RG20 hybridized to sites near the ends of the long arm of chromosome 7, and the short arm of chromosome 8, respectively, while the genomic clones RG28 and RG365 hybridized to sites near the ends of the long arm of chromosome 8. The genomic clone RG353 hybridized to a site near the end of the short arm of chromosome 11. Genomic clones RG190, R181, and RG361 hybridized to a site near the end of the short arm, while RG98 hybridized to a site near the end of the long arm of chromosome 12 (Fig. 2d–i, k–n Table 1). Even though the arms of all the chromosomes are very close to being metacentric, by using late-prophase and early-metaphase chromosome preparations, it was possible to distinguish the short from the long arms of all four chromosomes, as can be seen from the arm ratios listed in Table 1 and Fig. 2. It also merits comment that the position of the above genomic clones seems to be physically near the ends of the chromosomes. The labeled spots were all associated with the flanks of the chromatid near the end, rather than exactly on the end.

RG29 was originally mapped by McCouch et al. (1988) and Tanksley et al. (1989) to the linkage group for chromosome 7. The present study found it to hybridize to the short arm of chromosome 8. This apparent conflict was cleared up by Tanksley (1992) when the new Cornell linkage map placed RG29 on the linkage group associated with chromosome 8. However, RG98, shown by McCouch et al. (1988) to be located on the chromosome 12 linkage group, was also shown in the present study to hybridize to a site on chromosome 12 whereas the 1992 Cornell map has it located on the end of the chromosome-11 linkage group. There are several possible reasons for the observed differences. First, the 1988 and 1992 Cornell maps involve different mapping populations, which can result in changes in clone location. Second, whereas the present results show RG98 to hybridize to a site on chromosome 12 of rice variety IR36, it could hybridize to a different chromosome in another variety because of the occurrence of chromosomal interchanges. Finally, because of the similarity in size of chromosomes 11–12 we could have mis-mapped RG98 to the wrong chromosome. This is possible but unlikely since in three different studies spanning a period of 5 years no false hybridization sites have been detected in analyzing over 5 000 cells involving 50 rice clones.

The genomic clones RG4 RG170 hybridized to sites midway along the long arm of chromosome 7, where the average percent distances and their standard deviations were 45.67 ± 6.29 and 64.79 ± 5.87 (Figs. 1, 2 b–c, Table 1). The genomic clones RG118 and RG165 hybridized to

Table 1 Arm ratios and RFLP location averages for the chromosomes in the rice genome

RFLP	No. of detections	Ratio of long to short arm	RFLP location (%)[a] and SA or LA[c]	
Chromosome 7				
RG 165	11	1.21 ± 0.05[b]	~0	LA
RG4	8	1.12 ± 0.05	45.67 ± 6.29	LA
RG 170	6	1.19 ± 0.04	64.79 ± 5.87	LA
RG 30	7	1.17 ± 0.06	~100	LA
Chromosome 8				
RG 29	7	1.11 ± 0.03	~100	SA
RG 20	9	1.08 ± 0.01	~100	SA
RG 28	7	1.08 ± 0.02	~100	LA
RG 365	8	1.17 ± 0.03	~100	LA
Chromosome 11				
RG 353	11	1.14 ± 0.03	~100	SA
RG 118	8	1.14 ± 0.03	~0	LA
Chromosome 12				
RG 190	11	1.16 ± 0.04	~100	SA
RG 181	7	1.18 ± 0.06	~100	SA
RG 361	15	1.11 ± 0.03	~100	SA
RG 98	8	1.22 ± 0.02	~100	LA

[a] Percent distance from the centromere
[b] Standard deviation
[c] SA or LA, Short or long arm

sites at the region near the centromere on the long arm of chromosomes 11 and 7 respectively (Figs. 1, 2j and 2a, Table 1), which makes them the first clones in either rice, wheat, or barley to physically map to a centromeric region. Figure 2 shows that there was little or no background labeling and all of the hybridization signals were clear.

The signals were found on one chromatid of a homologue in each labeled cell except in one cell labeled by the genomic clone RG361 which showed hybridization sites on each chromatid of a single chromosome. This situation is not uncommon (Clark et al. 1989; Viegas-Pequignot et al. 1989). As indicated, the level of detection was 5.91%; therefore, the expected percentage for the simultaneous detection of the two spots would be very low.

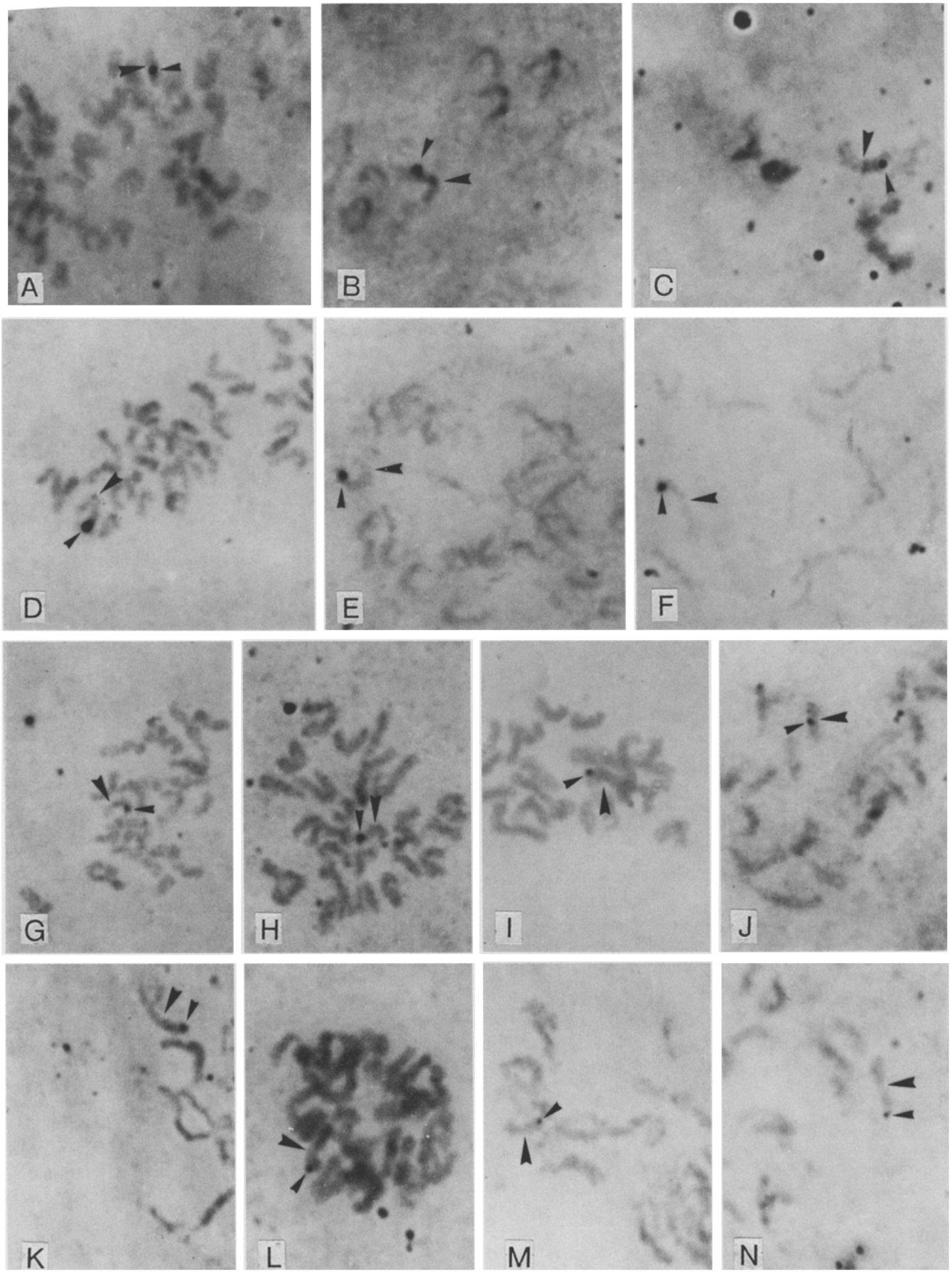
The average arm ratios of the chromosomes labeled by each genomic clone were very close to those of the corresponding chromosomes in the karyotype (Fig. 1) which was constructed according to the data from Kurata (1986). The average arm ratios of chromosomes labeled by two genomic clones in the same RFLP linkage group, for example, RG20 and RG28, or RG353 and RG118, were the same or almost the same. The chromosomes labeled by RG361 and RG98 show a difference in average arm ratio of 0.11 (Table 1), which is not unusual. Standard deviations ranged from 0.01 to 0.06 (Table 1), indicating that all measurements for the chromosomes labeled by the same genomic clone were taken from the same rice chromosome. Therefore, the present results were consistent with the data for the chromosomal location of the RFLP linkage groups (McCouch et al. 1988; Tanksley et al. 1989).

Comparable to the results for chromosomes 1 and 5 that Gustafson and Dillé (1992) reported, each of two terminal RFLP markers for chromosomes 8, 11–12 spanned a centromere and were distributed on both chromosome arms, while that from chromosome 7 was not ascertained. Each of two markers in chromosomes 8 and 12 hybridized to sites near the ends of both arms. This demonstrated that the RFLP markers of the linkage group convered a wide region of the chromosome's physical length. The linkage group for chromosome 11 contained 11 RFLPs covering 118 cM, while the linkage group for chromosome 12 contained 8 RFLPs covering 72 cM (Fig. 1). The physical distance covered by the linkage group for chromosome 11 spanned only the region of the long arm near the centromere to the region near the end of the short arm, but the distance covered by the linkage group for chromosome 12 extended from the region near the end of the long arm to that near the end of the short arm. This means that the greatest recorded cM distance was not the largest in terms of the physical distance covered on the chromosome and that there was some variation between genetic and physical maps. This agrees with other reports (Lucchesi and Suzuki 1968; Singh and Shepherd 1984; Dooner et al. 1985; Dooner 1986; Meagher et al. 1988; Gustafson et al. 1990a, b; Gustafson and Dillé 1992; Lukaszewsk and Curtis 1993) in both plants and animals. When studying rice, Gustafson and Dillé (1992) stated that these differences could be due to the varying distribution of heterochromatin blocks, the presence of recombinational "cold" spots, and different states of DNA condensation on the chromosomes. All of these could be relevant factors in causing the differences, but the actual reason may be more complicated and remains to be elucidated.

Fig. 2a–n **a** Early metaphase chromosomes showing a hybridization site with probe RG 165 on the long arm of chromosome 7 (Note: in this and all other Figs. the *small arrow* denotes the hybridization site and the *large arrow* denotes the centromere). **b** Late prophase chromosomes showing a hybridization site with probe RG 4 on the long arm of chromosome 7. **c** Early metaphase chromosomes showing a hybridization site with probe RG 170 on the long arm of chromosome 7. **d** Early-metaphase chromosomes showing a hybridization site with probe RG 30 on the long arm of chromosome 7. **e** Late prophase chromosomes showing a hybridization site with probe RG 29 on the short arm of chromosome 8. **f** Early-metaphase chromosomes showing a hybridization site with probe RG 20 on the short arm of chromosome 8. **g** Early-metaphase chromosomes showing a hybridization site with probe RG 28 on the long arm of chromosome 8. **h** Early-metaphase chromosomes showing a hybridization site with probe RG 365 on the long arm of chromosome 8. **i** Early-metaphase chromosomes showing a hybridization site with probe RG 353 on the short arm of chromosome 11. **j** Early-metaphase chromosomes showing a hybridization site with probe RG 118 on the long arm of chromosome 11. **k** Late-prophase chromosomes showing a hybridization site with probe RG 190 on the short arm of chromosome 12. **l** Early-metaphase chromosomes showing a hybridization site with probe RG 181 on the short arm of chromosome 12. **m** Early metaphase chromosomes showing a hybridization site of probe RG 361 on the short arm of chromosome 12. **n** Late-prophase chromosomes showing a hybridization site of probe RG 98 on the long arm of chromosome 12

It is clear that the lengths of recombinational maps can differ from the physical location of the probes. Part of this problem could be due to the fact that most molecular genetic maps have been derived from crosses involving distantly-related parents. It is widely known that, in cereals, crosses involving distantly-related parents can and do show a level of reduced meiotic pairing, which results in misdivision and even in an increase in the frequency of aneuploids. These factors can lead to an error in the calculation of genetic distances (Dvorak and Chen 1984; Curtis et al. 1991). Unfortunately, many species show drastically-reduced levels of polymorphism when closely-related parents are used, which makes for great difficulties in genetic mapping.

In situ hybridization can only give a close estimate of the physical location of a DNA sequence, because the labeled sites are capable of being large enough to cover up to a million basepairs. Another reason may be that different regions of the chromosomes have varying states of condensation during the mitotic process. Parts of the heterochromatin may pass through the cell cycle with relatively little change in the degree of condensation, while euchromatin exists in different states of condensation in different mitotic stages (Levin 1990). Kurata (1986), Dillé et al. (1990), Fukui and Iijima (1991) and others have shown that rice chromosomes appeared to condense differently from one part of the chromosome to another during the early stages of mitosis. It would be a serious mistake to assume that the DNA is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coiled, and distributed, equally on every chromosome of any given species. Therefore, it is possible that the percent distance of a given hybridization site from the centromere will still show some variation in different mitotic stages both within and between chromosomes and species. The genomic clone RG4 was located midway on the long arm of chromosome 7, with the shortest percent distance from the centromere to a hybridization site being 40%, and the longest being 57.15%, giving an average percent distance of 45.67% ± 6.29 (Table 1). The hybridization results of other probes showed that different cells labeled by the same probe had some hybridization sites very close to the end of the chromosome, while others were not quite at the end of the same chromosome. This may be due to the chromosomes sampled not being at the same mitotic stage. It is well known that the condensation state of chromosomes is not the same in different mitotic stages and may even vary within the same stage.

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