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Resolution of fluorescence *in-situ* hybridization mapping on rice mitotic prometaphase chromosomes, meiotic pachytene chromosomes and extended DNA fibers

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Abstract

Fluorescence *in-situ* hybridization (FISH) is a quick and affordable approach to map DNA sequences to specific chromosomal regions. Although FISH is one of the most important physical mapping techniques, research on the resolution of FISH on different cytological targets is scarce in plants. In this study, we report the resolution of FISH mapping on mitotic prometaphase chromosomes, meiotic pachytene chromosomes and extended DNA fibers in rice. A majority of the FISH signals derived from bacterial artificial chromosome (BAC) clones separated by approximately 1 Mb of DNA cannot be resolved on mitotic prometaphase chromosomes. In contrast, the relative positions of closely linked or even partially overlapping BAC clones can be resolved on a euchromatic region of rice chromosome 10 at the early pachytene stage. The resolution of pachytene FISH is dependent on early or late pachytene stages and also on the location of the DNA probes in the euchromatic or heterochromatic regions. We calibrated the fiber-FISH technique in rice using seven sequenced BAC clones. The average DNA extension was 3.21 kb/μm among the seven BAC clones. Fiber-FISH results derived from a BAC contig that spanned 1 Mb DNA matched remarkably to the sequencing data, demonstrating the high resolution of this technique in cytological mapping.

Introduction

The FISH technique, developed by Langer-Safer *et al.* (1982), is an important tool for physical mapping. FISH mapping played a significant role in the development of an integrated physical and cytological map of the human genome (Cheung *et al.* 2001). FISH mapping on human metaphase spreads permits rapid chromosomal localization of probes with a resolution of a 1–3 Mb (Lawrence *et al.* 1990, Lichter *et al.* 1990), whereas FISH on interphase nuclei can be used to order probes in the range of 50–1000 kb (Trask *et al.* 1989, van den Engh *et al.* 1992). The most recent development of FISH using extended DNA fibers has dramatically improved the resolving power for ordering probes that are

separated by as little as a few kb (Heng *et al*. 1992, Wiegant *et al*. 1992; for review, see Weier 2001).

FISH has become an increasingly popular technique for the physical mapping in plants (Jiang & Gill 1994, de Jong *et al*. 1999). The FISH technique is a quick and relatively affordable approach and complements other expensive or time-consuming physical mapping techniques that utilize cytogenetic stocks or DNA contig development. FISH techniques based on mitotic and meiotic chromosomes, interphase nuclei, synaptonemal complexes, and extended DNA fibers have been developed in plant species (Leitch *et al*. 1991, Albini & Schwarzacher 1992, Jiang *et al*. 1995, 1996, Xu & Earle 1996, Zhong *et al*. 1996, Fransz *et al*. 1996, Jackson *et al*. 1999, Peterson *et al*. 1999). As an additional cytological target compared to mammalian species, the meiotic pachytene chromosomes of plants are particularly useful for FISH mapping. Not only do the pachytene chromosomes provide superior mapping resolution compared to somatic metaphase chromosomes, but the euchromatin and heterochromatin features can be visualized on pachytene chromosomes, thereby allowing DNA probes to be mapped to specific euchromatic or heterochromatic regions (Zhong *et al*. 1999, Fransz *et al*. 2000, Cheng *et al*. 2001a).

Although the number of FISH reports has increased significantly in the last few years, research focusing on the resolution of FISH mapping techniques is rare in plants. In this report, we took advantage of the nearly complete sequencing of rice chromosome 10 and selected a number of sequenced BAC clones for comparison of FISH resolution on different cytological targets. The merits and pitfalls of each FISH mapping technique are discussed.

Materials and methods

Materials

Oryza sativa spp. *japonica* rice var. Nipponbare was used for both chromosome and DNA fiber preparation. All BAC clones used for FISH mapping were derived from the Nipponbare BAC libraries (http://www.genome.clemson.edu/orders/Product.html) and are anchored on chromosome 10 of rice (http://www.tigr.org/tigr-scripts/e2k1/bac_status_display.spl?db=osa1&status=All&chromosome=10&seqdb=osg).

Chromosome preparation

Rice root tips were harvested from germinated seeds or plants growing in the field, pretreated in 0.002 mol/L 8-hydroxyquinoline at 20°C for 2 h to accumulate prometaphase cells, and fixed in methanol : acetic acid (3 : 1). Root tips were macerated in 2% cellulose and 1% pectinase at 37°C for 1.5 h, and squashes were made in the same fixative. Young panicles of rice were harvested and fixed in 3 : 1 (100% ethanol : glacial acetic acid) Carnoy's solution. Microsporocytes at the pachytene stage were squashed in 45% acetic acid. Slides were stored at − 80°C until use. After removing the coverslips, slides were dehydrated through an ethanol series (70%, 90% and 100%) prior to use in FISH.

FISH on chromosomes

The FISH procedure applied to both mitotic and meiotic chromosomes was essentially the same as previously published protocols (Jiang *et al*. 1995). Biotin-labeled and digoxigenin-labeled probes were detected using a fluorescein isothiocyanate (FITC)-conjugated anti-biotin antibody (Vector Laboratories, Burlingame, CA) and a rhodamine-conjugated anti-digoxigenin antibody (Roche Diagnostics, Indianapolis, IN), respectively. Chromosomes were counter-stained with DAPI in an antifade solution Vectashield (Vector Laboratories, Burlingame, CA).

FISH on DNA fibers

Leaf nuclei were prepared as previously described (Jackson *et al*. 1998). A suspension of nuclei (2 μl) was deposited at one end of a poly-L-lysine slide (Sigma, St. Louis, MO) and permitted to air dry for 5–10 min. Eight microlitres of STE lysis buffer (STE: 0.5% (w/v) SDS, 5 mmol/L EDTA, 100 mmol/L Tris, pH 7.0) was pipetted on top of the nuclei and the slide was incubated at room temperature for 4 min. A clean coverslip was used to slowly drag the solution down the slide. The preparation was air dried, fixed in 3 : 1 ethanol : glacial acetic acid for 2 min, and baked at 60°C for

30 min. The probe mixture (20 µl) was applied to the DNA fiber preparation and covered with a 22×40 mm coverslip and sealed with rubber cement. The slide was placed in an 80°C oven in direct contact with a heated surface for 3 min, transferred to a wet chamber, which was prewarmed in an 80°C oven, for 2 min, and transferred to 37°C for overnight incubation. Post-hybridization washing stringency was the same as FISH on chromosome spreads. Signal detection was according to Jackson *et al.* (1998).

Cytological measurements and analysis

All images were captured digitally using a SenSys CCD (charge coupled device) camera (Photometrics, Tucson, AZ) attached to an Olympus BX60 epifluorescence microscope. The CCD camera was controlled using IPLab Spectrum v3.1 software (Signal Analytics, Vienna, VA) on a Macintosh computer. Gray scale images were captured for each color channel and then merged. Measurements were made on the digital images of the FISH signals and chromosomes within IPLab Spectrum software and final image adjustments were done with Adobe Photoshop 5.1. A minimum of ten data points were collected to map the BAC clones on the DNA fibers.

Results

FISH resolution on prometaphase chromosomes

We used rice prometaphase chromosomes for FISH mapping because rice metaphase chromosomes are only 1–3 µm in size and are too small for mapping purposes. In order to determine the limits of the FISH resolution on rice prometaphase chromosomes, we selected four pairs of BAC clones for FISH analysis. These four pairs of BAC clones are separated by different distances (Table 1) and are all located in the subtelomeric region of the long arm of rice chromosome 10. BACs E64P21 and 10C11 are separated by 0.69 Mb (not including the two BAC inserts) with E64P21 oriented towards the centromere and 10C11 towards the telomere. The majority of the FISH signals derived from these two BACs were either partially or completely overlapping on prometaphase chromosomes (Figure 1A). The telomere–centromere orientation of the two BACs could be determined in only 26% of the partially overlapping signals (Table 1). A reverse orientation of the green/red signals was occasionally observed. When the distances between two BACs are increased to 0.81 Mb, 2.22 Mb and 3.74 Mb, the correct telomere–centromere orientation could be resolved 35%, 52%, and 84% of the time, respectively, of the targeted chromosomes (Table 1).

FISH on pachytene chromosomes

Rice meiotic pachytene chromosomes are nearly 20 times as long as rice metaphase chromosomes (Cheng *et al.* 2001c). Thus, the highly extended pachytene chromosomes provide a superior resolving power for FISH mapping as compared to somatic metaphase chromosomes. We previously demonstrated that the telomere–centromere orientation of BAC clones that are separated by less than 100 kb could be resolved on rice pachytene chromosomes (Cheng *et al.* 2001b). In this study, we selected several chromosome 10 BACs to further analyze the resolving power of pachytene FISH.

Table 1. Telomere–centromere orientation of FISH signals derived from pairs of BAC clones on rice prometaphase chromosomes.

BAC pairs	Distance (Mb)[1]	Chromosomes observed	No. chromosomes with correct signal orientation	No. chromosomes with no signal orientation[2]	No. chromosomes with reversed signal orientation
E64P21–10C11	0.69	50	13 (26%)	36 (72%)	1 (2%)
E18B10–E64P21	0.81	46	16 (35%)	28 (61%)	2 (4%)
41P03–E64P21	2.22	50	26 (52%)	24 (48%)	0 (0%)
93B11–10C11	3.74	44	37 (84%)	7 (16%)	0 (0%)

[1]The two BAC inserts are not included in the distance. These distances are calculated using available sequence and fingerprint data.
[2]Signals from the two BACs overlapped completely and their telomere–centromere orientation could not be determined.

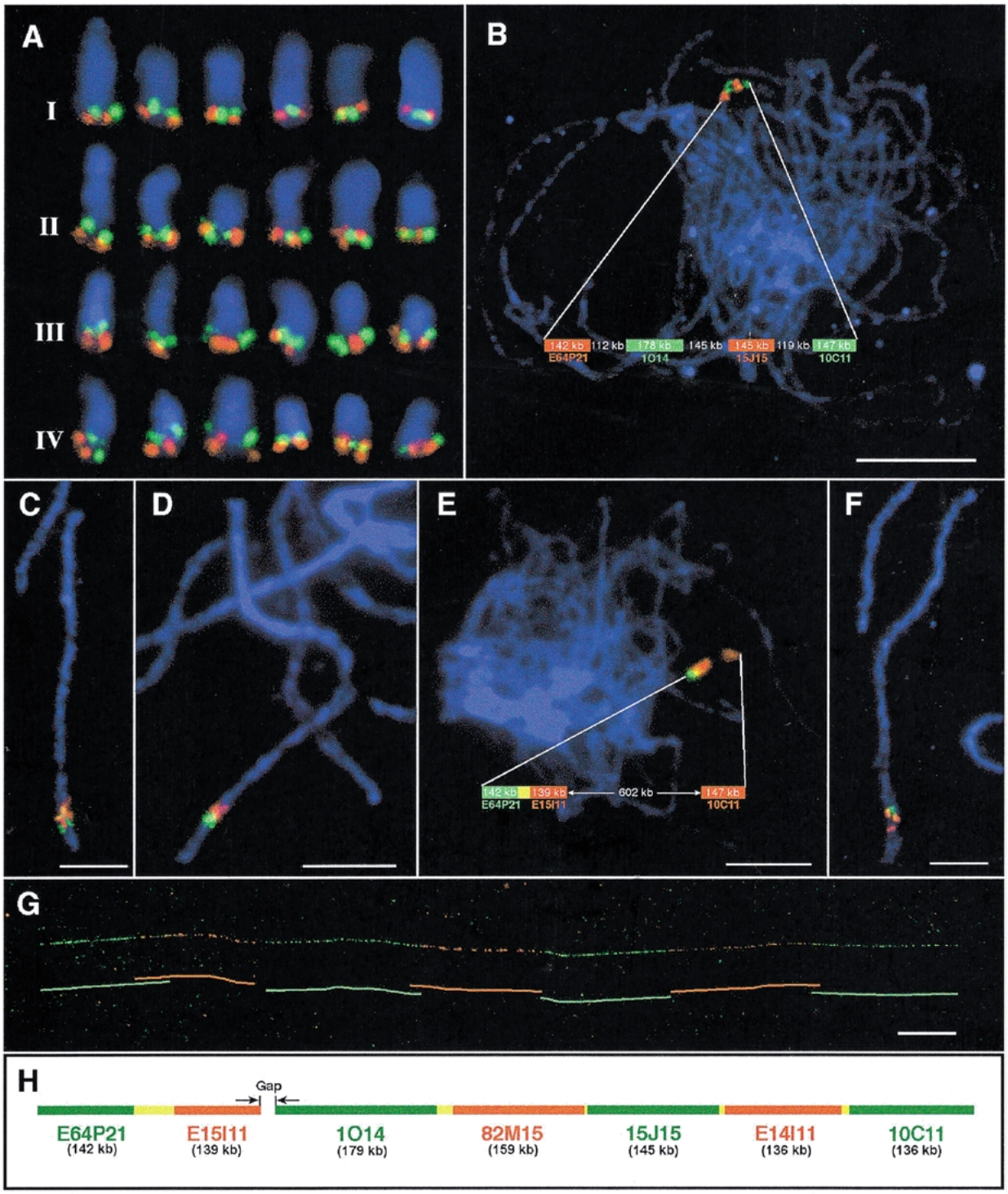
A
I
II
III
IV
B
142 kb
E64P21
112 kb
178 kb
1O14
145 kb
145 kb
15J15
119 kb
147 kb
10C11
C
D
E
142 kb
E64P21
139 kb
E15I11
602 kb
147 kb
10C11
F
G
H
Gap
E64P21
(142 kb)
E15I11
(139 kb)
1O14
(179 kb)
82M15
(159 kb)
15J15
(145 kb)
E14I11
(136 kb)
10C11
(136 kb)

Figure 1B, C demonstrates that the FISH signals derived from four BAC clones separated by 112–145 kb are well separated on early pachytene chromosomes, but the FISH signals derived from these BAC clones tended to be partially overlapping on late pachytene chromosomes. BACs E15I11 (139 kb) and 1O14 (178 kb) are separated only by ~12 kb based on fiber-FISH results. Although the FISH signals derived from these two BAC clones overlapped (Figure 1D), the centromere/telomere orientation of these two BACs could be determined on many late pachytene chromosomes. BACs E64P21 and 15I11 overlap by 38 kb and the relative positions of these two BAC clones could also be resolved on early pachytene chromosomes (Figure 1E) but were difficult to resolve on late pachytene chromosomes (Figure 1F).

The distance between the FISH signals derived from BAC clones E64P21 and 10C11, which span approximately 1 Mb (Table 2), was measured on 20 pachytene chromosomes. The average distance was 3.17 μm (2.99–3.53, eight measurements) on early pachytene chromosomes and 2.26 μm (1.82–2.45, 12 measurements) on late pachytene chromosomes. These distances can be converted to a DNA content of 315 kb/μm of early pachytene chromosomes and 442 kb/μm of late pachytene chromosomes in this euchromatic region.

FISH on extended DNA fibers

The fiber-FISH technique in plants has only been calibrated in *Arabidopsis thaliana* (Fransz *et al.* 1996, Jackson *et al.* 1998). The extension degree of DNA fibers prepared from *A. thaliana* was reported as 3.27 kb/μm by Fransz *et al.* (1996) and 2.87 kb/μm by Jackson *et al.* (1998). We selected a ~1 Mb contig that contains seven rice BAC clones (Table 2). All of these BACs have been sequenced and are located in the sub-telomeric region on the long arm of chromosome 10 [E64P21 (AC073166), E15I11 (AC051633), 1O14 (AC025783), 82M15 (AC020666), 15J15 (AC026758), E14I11 (AC037426), and 10C11 (AC069300)]. The seven BAC clones were analyzed in pairs by FISH on DNA fibers prepared from Nipponbare. The microscopic sizes (μm) of the fiber-FISH signals derived from each BAC and the overlapping region of adjacent BACs were measured using IPLab Spectrum software. The correlation between the microscopic size of the fiber-FISH signal and the insert size among the seven BACs ranged from 3.02 kb/μm (BAC E15I11) to 3.50 kb/μm (BAC 82M15) with an average of 3.21 kb/μm, suggesting limited variation of DNA fiber extension in the experiments. The fiber-FISH measurements of the physical sizes of the BAC inserts and their overlapped regions matched remarkably to the sequencing data (Table 2). Sequencing results showed BAC E15I11 and BAC 1O14 do not overlap and this physical gap was confirmed by fiber-FISH analysis and was estimated as 11.6 ± 4.4 kb. Figure 1G shows a fiber-FISH signal derived from an alternate green/red labeling using all seven BACs.

Discussion

Sensitivity and resolution are the two most important parameters of the FISH mapping tech-

Figure 1. FISH mapping on chromosomes and extended DNA fibers in rice. (**A**) Selected prometaphase chromosomes prepared from the root tips of Nipponbare were probed with two BAC clones that are separated by 0.69 Mb (row I), 0.81 Mb (row II), 2.22 Mb (row III) and 3.74 Mb (row IV), respectively. (**B**) An early pachytene cell probed with four BAC clones separated by between 112 kb and 145 kb. The signals from the four BACs are well separated from each other. (**C**) The same four BACs as in (**B**) on a late pachytene chromosome 10. The signals are partially overlapped and are not unambiguously resolved. (**D**) FISH signals derived from BAC E15I11 (red) and 1O14 (green), which are separated by ~12 kb based on fiber-FISH results. (**E**) FISH mapping on early pachytene chromosomes using BACs E64P21 (green) and E15I11 (red), which are overlapped by 38 kb, and 10C11 (red) that is 602 kb away from E15I11. *Note*: the orientation of the overlapping signals derived from E64P21 and E15I11 can be resolved. (**F**) The same three BACs as in (**E**) on a late pachytene chromosome 10. The signals derived from E64P21 and E15I11 signals are almost completely overlapped and their orientation cannot be unambiguously resolved. (**G**) Fiber-FISH analysis of a ~1 Mb contig that includes seven BAC clones. The seven BACs were detected by alternative green/red colors. Diagrammatic signals were drawn based on the true signals derived from each BAC. (**H**) Diagram of the ~1 Mb BAC contig used in fiber-FISH. The green/red color bars of the seven BACs correspond to the fiber-FISH signals in (**G**). The yellow bars represent the overlapping regions between adjacent BACs. The scale for the diagram was drawn based on sequence data. The size of the gap was drawn according to the fiber-FISH data. Bars represent 10 μm in **B**, 20 μm in **G**, and 5 μm in **C**, **D**, **E**, and **F**.

Table 2. Comparison of fiber-FISH and sequencing data on a ~1 Mb BAC contig.

	Fiber-FISH data					Sequencing data[2]	
BAC	FISH signal (μm)	Overlap signal (μm)	*n*	Insert size[1] (kb)	Overlap size[1] (kb)	Insert size (kb)	Overlap or gap (kb)
E64P21	45.51 ±7.36		10	144.8 ±23.4		142.1	
		10.58 ±2.69	10		33.7 ±8.6		38.3
E15I11	45.89 ±9.51		20	146.0 ±30.3		138.8	
		3.63 ±1.39 (gap)	10		11.6 ±4.4 (gap)		gap
1O14	58.41 ±8.60		20	185.9 ±27.4		178.0	
		5.33 ±1.74	10		16.9 ±5.5		9.8
82M15	45.97 ±8.21		20	146.3 ±26.1		158.6	
		0.79 ±0.31	10		2.5 ±1.0		3.5
15J15	44.70 ±8.92		20	142.3 ±28.4		144.8	
		1.59 ±0.26	10		5.1 ±0.8		6.9
E14I11	43.00 ±7.61		20	136.8 ±24.2		135.5	
		4.87 ±1.22	10		15.2 ±3.8		9.4
10C11	42.02 ±8.45		10	133.7 ±26.9		147.1	

[1] The physical sizes (kb) of the BAC inserts and their overlap or gap were converted on the basis of the average DNA extension degree of 3.21 kb/μm (1044.9 kb/325.5 μm) of the seven BACs.
[2]The GenBank accession numbers for the BACs are E64P21 (AC073166), E15I11 (AC051633), 1O14 (AC025783), 82M15 (AC020666), 15J15 (AC026758), E14I11 (AC037426), and 10C11 (AC069300).

niques. Various procedures have been used to improve the sensitivity of the FISH techniques in plant species, allowing for an increasingly improved frequency of detection of small DNA probes on plant chromosomes. However, research investigating the resolution of FISH mapping has been rare. The resolution of FISH depends on the cytological targets, which include interphase nuclei, mitotic prometaphase/metaphase chromosomes, meiotic pachytene chromosomes and extended DNA fibers, and the stages of mitotic and meiotic divisions, in which the chromosomes have different degrees of condensation.

The mitotic metaphase chromosomes in plant species are highly condensed and offer very low resolution for FISH mapping (Pedersen & Linde-Laursen 1995, Desel *et al.* 2001). The minimum physical distance that would be required to resolve the telomere–centromere orientation of two DNA probes on plant metaphase chromosomes is not known. Pedersen & Linde-Laursen (1995) suggested that a minimum of 5–10 Mb would be necessary to resolve DNA clones on barley metaphase chromosomes, which is considerably larger than the 1–3 Mb of minimum distance to resolve clones on human metaphase spreads (Lawrence *et al.* 1990). In this study, we demonstrated that the majority of the signals derived from two BAC clones separated by less than 1 Mb overlap completely on rice prometaphase chromosomes. About half of the signals derived from two BACs separated by 2 Mb overlap completely on prometaphase chromosomes. These results show that a large number of FISH signals need to be analyzed in order to judge the centromere–telomere orientation of two DNA clones separated by less than 2 Mb.

The BAC clones selected in this study are located at the subtelomeric region of the long arm of chromosome 10. Because distal regions are generally more decondensed compared to the proximal regions on rice prometaphase chromosomes, we expect that the minimum physical distance required to resolve two BACs will be substantially larger than 1 Mb in the proximal regions of prometaphase chromosomes and in the more condensed metaphase chromosomes. Despite its low resolution, the readily available root tip tissue makes mitotic prometaphase/metaphase chromosome-based FISH mapping an easy and quick approach to determine the chromosomal location of DNA probes.

Pachytene chromosome-based FISH mapping provides an intermediate resolution between fiber-

FISH and FISH on mitotic chromosomes. The resolution of pachytene FISH depends on early or late stages of individual pachytene cells. Early pachytene chromosomes of rice can be twice as long as late pachytene chromosomes. In a previous study, we demonstrated that DNA clones separated by 40 kb could be resolved on early pachytene chromosomes but not on late pachytene chromosomes (Cheng *et al.* 2001b). The higher resolution of FISH on early rather than late pachytene chromosomes is also demonstrated in this study (Figure 1B, C, E, F). Even partially overlapped BAC clones could be resolved on early pachytene chromosomes (Figure 1E). The disadvantage of using early pachytene chromosomes is that the chromosomes are difficult to separate from each other which often prevents the localization of the centromere and telomeres of the targeted chromosome.

The resolution of pachytene FISH is also dependent on the location of the targeted DNA clones in the heterochromatic or euchromatic regions. Information on pachytene FISH resolution within different types of chromatin is scarce in the literature. de Jong *et al.* (1999) reported that pachytene FISH can resolve 1.2 Mb in heterochromatin and 120 kb in euchromatin in tomato. In contrast, in *A. thaliana* resolution of the order of 60 kb and 140 kb has been suggested for euchromatin and heterochromatin, respectively (de Jong *et al.* 1999). The BAC clones used in this study were derived from a euchromatic region on the long arm of rice chromosome 10. Even partially overlapping BAC clones from this region can be resolved at the early pachytene stage. We observed a significant variation in the intensity of DAPI staining on individual rice pachytene chromosomes (Cheng *et al.* 2001c), indicating that the heterochromatic regions on the 12 chromosomes may have different degrees of condensation at the pachytene stage. Thus, the resolution of pachytene FISH in the heterochromatic regions on different chromosomes may vary significantly. The short arm of rice chromosome 10 is highly heterochromatic as compared to the long arm (Cheng *et al.* 2001c). FISH mapping of BAC clones separated by a similar distance showed different resolution on the two arms of chromosome 10 (Cheng *et al.* 2001b) but not a 10-fold difference as reported in tomato (de Jong *et al.* 1999), suggesting that the rice heterochromatin may not be as condensed as the pericentromeric heterochromatin in tomato.

DNA fiber-based FISH provides the highest mapping resolution of all cytological mapping techniques. This technique has been used in plants to analyze the structure of repetitive DNA sequence families (Fransz *et al.* 1996, Dong *et al.* 1998, Jackson *et al.* 1998, Miller *et al.* 1998, Pich & Schubert 1998, Zhong *et al.* 1998, Ohmido *et al.* 2000, Fukui *et al.* 2001, Gindullis *et al.* 2001), to analyze transgenic DNA (Wolters *et al.* 1998; Jackson *et al.* 2001, Svitashev & Somers 2001), to measure physical distances between DNA clones (Jackson *et al.* 1998, 2000, Cheng *et al.* 2001b, Stupar *et al.* 2001), and to analyze complex DNA molecules (Jackson *et al.* 1999, Lilly *et al.* 2001). To calibrate the fiber-FISH technique in rice, we measured the DNA extension degree using seven sequenced BAC clones covering ~1 Mb of contiguous DNA. The relationship between the microscopic size and the sequenced insert size among the seven BAC clones averaged 3.21 kb/μm, which is between the two calibration numbers, 2.87 and 3.27 kb/μm, reported in *A. thaliana* (Fransz *et al.* 1996, Jackson *et al.* 1998). Fiber-FISH analysis of a seven-BAC contig with 10–20 measurements from each BAC showed a remarkable correlation between the cytological and the sequencing data (Table 2). Our results demonstrated that the variation in the fiber-FISH measurements is significantly smaller on larger molecules compared with smaller molecules. DNA probes less than 10 kb generate only a few consecutive fluorescence spots resulting in large variation. This variation can be minimized by analyzing a large number of signals. A small gap in the middle of the analyzed contig (Figure 1G) was confirmed by fiber-FISH mapping. These results, together with previous work on *A. thaliana* (Jackson *et al.* 1998), demonstrate that fiber-FISH is a very efficient and powerful technique for confirming BAC contig maps and in gauging the physical sizes of large sequencing gaps.

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