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Metaphase and interphase fluorescence *in situ* hybridization mapping of the rice genome with bacterial artificial chromosomes

(physical mapping/genomic DNA clones)

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ABSTRACT **Fluorescence *in situ* hybridization (FISH) is a powerful tool for physical mapping in human and other mammalian species. However, application of the FISH technique has been limited in plant species, especially for mapping single- or low-copy DNA sequences, due to inconsistent signal production in plant chromosome preparations. Here we demonstrate that bacterial artificial chromosome (BAC) clones can be mapped readily on rice (*Oryza sativa* L.) chromosomes by FISH. Repetitive DNA sequences in BAC clones can be suppressed efficiently by using rice genomic DNA as a competitor in the hybridization mixture. BAC clones as small as 40 kb were successfully mapped. To demonstrate the application of the FISH technique in physical mapping of plant genomes, both anonymous BAC clones and clones closely linked to a rice bacterial blight-resistance locus, *Xa21*, were chosen for analysis. The physical location of *Xa21* and the relationships among the linked clones were established, thus demonstrating the utility of FISH in plant genome analysis.**

Fluorescence *in situ* hybridization (FISH) has become an important *in situ* hybridization tool because of its amenability of coupling with highly sensitive charge coupled device (CCD) camera and digital imaging analysis and its potential for mapping of several probes simultaneously. In humans, DNA probes smaller than 1 kb have been mapped on metaphase chromosomes by FISH (1) and DNA fragments separated by as little as 50–100 kb have been ordered within interphase nuclei or on prophase chromosomes (2–4). Ried *et al.* (5) mapped seven probes simultaneously by using a combinatorial fluorescence and digital imaging analysis. The success of FISH analysis in humans has also been facilitated by the application of hybridization techniques that suppress cross-hybridization from repetitive DNA sequences in the genomic clones by preannealing with unlabeled human genomic DNA in the hybridization mixture. Thus, specific clones can be isolated from cosmid, P1, bacterial artificial chromosome (BAC), or yeast artificial chromosome (YAC) libraries and used directly for FISH mapping. Large numbers of genomic clones from chromosome-specific libraries have been used successfully to generate high-density cytological maps (6–8).

In plants, *in situ* hybridization techniques have been used mainly for mapping repetitive DNA sequences and multicopy gene families (9). Mapping of low or single-copy sequences has proven difficult compared to that in humans. In most of the successful *in situ* hybridization experiments in plants, the target DNA sequences on chromosomes were >10 kb long (10–14). Mapping of small probes was also reported (15); however, the frequency of signal detection was very low and the technique is not very reliable and reproducible (9). The relatively low sensitivity of *in situ* hybridization (including FISH) in plants is arguably due to the presence of rigid cell walls and cytoplasmic debris and the more pronounced condensation of plant metaphase chromosomes.

Because of the difficulty of using small single-copy sequence probes, genomic DNA cloned in large insert vectors should be a viable alternative approach to map genetic loci in plants. Even if repetitive sequences account for 90% of plant genomic DNA, a 100-kb genomic clone theoretically should contain ≈10 kb of unique sequence, enough to generate a good signal in most plant species examined to date. In the present work, we demonstrate the application of BAC clones for FISH mapping in rice (*Oryza sativa* L.).

MATERIALS AND METHODS

Rice BAC Clones. A rice BAC library was constructed from the rice line IR-BB21 containing *Xa21*, a bacterial blight disease-resistance locus derived from a wild African species, *Oryza longistaminata* (16). Five BAC clones linked to *Xa21* and five random clones (Table 1) from this library were used for FISH mapping. Five additional random clones from a different BAC library were kindly provided by R. A. Wing (Texas A&M University) (Table 1). The insert sizes of the mapped BAC clones range from 40 to 220 kb.

Strains. Rice lines DV85 and IR-BB21 were used in FISH mapping. DV85 is an indica rice cultivar. Line IR-BB21 is a nearly isogenic line (NIL) for *Xa21* that was constructed by backcrossing *O. longistaminata* (*Xa21* donor parent) five times to the recurrent parent IR24 followed by five selfings (18).

Chromosome Preparation. Seeds were germinated on moist filter paper in Petri dishes. Roots 1–2 cm long were cut and directly fixed in methanol/glacial acetic acid (3:1) for several hours up to a few days. The fixed root tips were washed thoroughly with 0.01 M citrate buffer (sodium citrate/citric acid, pH 4.8) and digested in 6% (wt/vol) cellulase (Calbiochem) and 2% (wt/vol) pectolyase (Sigma) for 30 min at 37°C. The enzymes were carefully washed from the softened material and replaced with 0.01 M citrate buffer for 10 min; the root tips were subjected to a hypotonic treatment in distilled water for 20 min and transferred to ethanol-washed glass slides. The tissue was macerated in a drop of methanol/glacial acetic acid (1:1) with a razor blade. The slides were air dried and stored in a −80°C freezer. The slides were treated with an ethanol series (70%, 90%, and 100% ethanol; 5 min each) just prior to *in situ* hybridization.

Probe Labeling. BAC DNA was isolated by an alkaline lysate method (19). The purified DNA was labeled by standard nick-translation reaction mixtures containing biotin (bio)-11-

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Abbreviations: bio, biotin; dig, digoxigenin; FISH, fluorescence *in situ* hybridization; CCD, charge coupled device; FITC, fluorescein isothiocyanate; DAPI, 4′,6-diamidino-2-phenylindole; PI, propidium iodide; RFLP, restriction fragment length polymorphism; YAC, yeast artificial chromosome; BAC, bacterial artificial chromosome; PFGE, pulsed-field gel electrophoresis; NIL, nearly isogenic line.

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Table 1. BAC clones used in the present study

Name	Insert size, kb	Description*	Interphase signals	Repetitive sequences†	Source
UCD103-1	100	RG103	2	ND	UC–Davis
UCD103-5	90	RG103	2	ND	UC–Davis
UCD103-7	120	RG103	2	ND	UC–Davis
UCD818-13	85	pTA818	2	ND	UC–Davis
UCD818-14	40	pTA818	2‡	ND	UC–Davis
UCD2	220	Random	2	–	UC–Davis
UCD6	140	Random	2	–	UC–Davis
UCD11	130	Random	2	–	UC–Davis
UCD16	140	Random	2	+	UC–Davis
UCD18	170	Random	2	+	UC–Davis
TQ7A1	100	Random	2	–	Texas A&M
TQ7A3	140	Random	2	–	Texas A&M
TQ7A4	135	Random	2 + 2 minor	+	Texas A&M
TQ7A5	130	Random	2	–	Texas A&M
TQ7A8	110	Random	2	–	Texas A&M

*Random clones or clones isolated using DNA markers RG103 and pTA818 (16, 17).
†–, Discrete signals were observed without competitive genomic DNA; +, discrete signals were observed only with competitive genomic DNA; ND, no data.
‡Two spots were observed in normal rice line DV85, while four spots were observed in IR-BB21.

dUTP or digoxigenin (dig)-11-dUTP, and labeled probes were purified by passage through Sephadex G-50 columns. Although most BAC DNA preparations contained some bacterial genomic DNA, this contaminating DNA does not interfere with the FISH analysis, indicating that *Escherichia coli* does not share enough homologous DNA with rice to give detectable FISH signals. For two-color FISH, two different probes were labeled with bio-11-dUTP and dig-11-dUTP, respectively, while in three-color FISH the third probe was labeled combinatorially with 50% bio-11-dUTP and 50% dig-11-dUTP.

***In Situ* Hybridization and Detection.** The *in situ* hybridization protocol was modified slightly from that of Rayburn and Gill (20). About 10 ng of labeled BAC DNA was used for each slide in a hybridization mixture with 50% formamide/10% dextran sulfate/2× SSC/10 μg of salmon sperm DNA/≈1 μg of Cot-1 fraction of rice genomic DNA. The mixture was denatured at 80°C for 10 min, centrifuged briefly, and preannealed at 37°C for 1 hr before applying to slides. Slide-bound chromosomal DNA was denatured in a solution of 70% formamide in 2× SSC for 1.5 min at 80°C and dehydrated in a −20°C ethanol series (70%, 90%, and 100% ethanol; 5 min each). Ten microliters of hybridization mixture was applied to each slide and sealed under a coverslip (18 × 18 mm) with rubber cement.

After overnight incubation at 37°C, the coverslips were removed and the slides were washed at room temperature in 2× SSC for 5 min, at 45°C in 2× SSC for 10 min, at room temperature in 2× SSC for 5 min, and at room temperature in 1× PBS (phosphate-buffered saline) for 5 min. The biotinylated probes were detected with fluorescein isothiocyanate-conjugated avidin (FITC–avidin) (Vector Laboratories) and the dig-labeled probes were detected with a rhodamine-conjugated anti-dig antibody (Boehringer Mannheim). 4′,6-Diamidino-2-phenylindole (DAPI) was used as a chromosome counterstain in multicolor FISH while propidium iodide (PI) was used as a chromosome counterstain in single-color FISH with biotinylated probes.

Digital Imaging. Images were obtained with a Zeiss Axioskop epifluorescence microscope coupled to a cooled CCD camera (PM512; Photometrics; Tucson, AZ). Camera control and digital image acquisition (8-bit gray scale) were implemented with an Apple Macintosh IIx computer. Fluorophores were selectively imaged with filter cubes especially prepared by Zeiss (filter 487910 for fluorescein, filter 487915 for rhodamine and PI, and filter 487901 for DAPI) to minimize image registration problems and merged as described (5). It is worth emphasizing that although a CCD imaging system was used, the signals of the probes were clearly visible by eye through the microscope.

RESULTS

Metaphase and Interphase Mapping of BAC Clones. To investigate whether the presence of the repetitive sequences in the BAC clones would generate high levels of nonspecific hybridization, FISH was performed on 10 random BAC clones in the absence of Cot-1 rice genomic DNA. Surprisingly, discrete chromosomal loci could be identified for 7 of the 10 clones. Although minor hybridization signals were distributed uniformly over all the chromosomes and nuclei, the stronger signals from the unique sequences of the BAC clones were clearly distinguishable from the background (Fig. 1*a*). However, the signal/noise contrast with these BACs was greatly improved in the presence of competitive rice genomic DNA. Distinct FISH signals were observed with the other three BAC clones only in the presence of the rice competitor DNA (compare Fig. 1 *b* and *c*). FISH signals were obtained from all the BACs analyzed, including clone 818-14 that contained a relatively small insert of ≈40 kb (Table 1).

Both metaphase chromosomes and interphase chromatin were scored in this FISH analysis. Clear signals were observed in 50–90% of the interphase nuclei and in >90% of the complete metaphase spreads. In most cases, signals were observed on both chromatids on each pair of homologous metaphase chromosomes, although merged signals from two sister chromatids were also observed frequently. As expected, prometaphase chromosomes generally gave better resolution (relative position on a specific chromosome arm) of the signals than the more condensed metaphase chromosome preparations.

Fourteen of the 15 BAC clones analyzed in line DV85 gave two FISH signals in interphase nuclei. This suggests that the inserts in these clones constitute a single piece of rice genomic DNA and thus are not chimeric. The remaining clone, TQ7A4, gave in addition to two major spots one or two minor spots in some of the interphase nuclei. We do not know whether these minor spots reflect a chimeric DNA insert or the presence of related sequences at a second locus in the genome.

We next evaluated the utility of multicolor FISH for mapping several probes simultaneously. Rice prometaphase chromosomes have different DAPI or PI staining intensities in different parts of the chromosomes due to uneven condensation. In general, for most of the chromosomes, there is a decreased staining toward the telomeres. This staining pattern

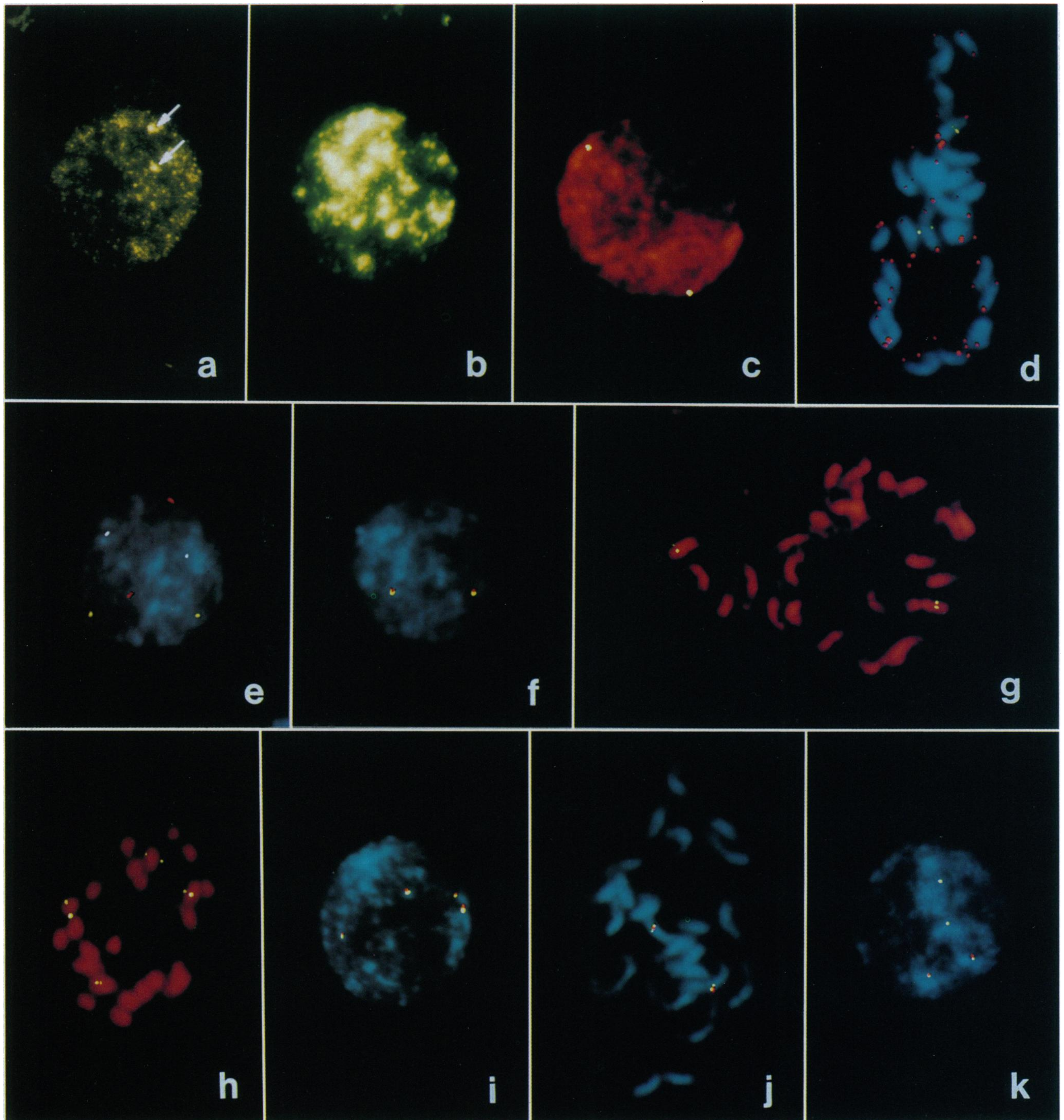

FIG. 1. (*a*) Interphase mapping of BAC clone TQ7A5. Cot-1 rice genomic DNA was not applied during hybridization. Two major spots (arrows) can be observed along with the minor spots all over the nucleus. The cell was not counterstained. (*b*) Interphase mapping of BAC clone UCD18. Cot-1 genomic rice DNA was not applied. No discrete signals can be observed. The cell was not counterstained. (*c*) Interphase mapping of UCD18. Cot-1 rice genomic DNA was applied during hybridization. Two discrete signals can be observed. The cell was counterstained by PI. (*d*) Cohybridization of BAC clone TQ7A1 (labeled with bio-11-dUTP and detected with FITC–avidin; yellow spots) with telomeric sequence probe pAtT4 (labeled with dig-11-dUTP and detected with rhodamine-conjugated anti-dig antibody; red spots). TQ7A1 is located on the proximal region of the long arm of a pair of unidentified chromosomes. (*e*) Interphase three-color FISH mapping of three random BAC clones: TQ7A5 (yellow), UCD2 (red), and UCD6 (white). (*f*) Interphase mapping of 103-1 (yellow) and 103-7 (red) in IR-BB21. Signals from the two clones overlap. (*g*) Metaphase mapping of the bacterial blight-resistance locus *Xa21* in IR-BB21 using a closely linked BAC clone 103-7. Signals are located at the middle of the long arm of chromosome 11. (*h*) Metaphase mapping of 818-14 in IR-BB21. Signals are located on two pairs of chromosomes. (*i*) Interphase mapping of three BAC clones in IR-BB21. Clone 818-13, labeled by bio-11-dUTP and detected by FITC, two relatively small yellow spots; clone 103-7, labeled by bio-11-dUTP and detected by FITC, two relatively large yellow spots; clone 818-14, labeled by dig-11-dUTP and detected by rhodamine, two small and two large red spots. Note: two small yellow spots overlap with two small red spots; two large yellow spots overlap with two large red spots. (*j*) Metaphase mapping of 818-13 (yellow) and 818-14 (red) in DV85. Signals from the two clones overlap. Signals are located at a proximal region of a pair of unidentified chromosomes. (*k*) Interphase mapping of three BAC clones in DV85: 818-13 (yellow), 103-7 (yellow), and 818-14 (red). Signals from 818-13 and 818-14 overlap. Signals from 103-7 are independent from those of 818-13 and 818-14.

makes it difficult to locate the true ends of the chromosomes and the relative chromosomal location of the probes. To address this problem, we cohybridized a telomeric sequence probe, pAtT4 derived from *Arabidopsis thaliana* (21), with specific BAC clones. Fig. 1*d* shows the cohybridization of BAC clone TQ7A1 with pAtT4. TQ7A1 is located on the proximal region of the long arm of a pair of unidentified chromosomes. Hybridization signals from pAtT4 are visible on both ends of this pair of chromosomes.

Fig. 1*e* demonstrates the interphase mapping of three different BAC probes by three-color FISH. The signals from the third probe were pseudocolored with white to distinguish it from the other two probes. Multicolor FISH was also used to analyze the relative position of physically closely related BAC clones (see below).

Physical Mapping of BAC Clones Linked to *Xa21*. A rice bacterial blight disease-resistance locus, *Xa21*, was transferred from a wild species, *O. longistaminata*, into cultivated rice (18, 22). Several closely linked DNA markers to *Xa21* were identified in NIL IR-BB21 (17). BAC clones were isolated by using these DNA markers as probes (16) and analyzed by FISH.

Three BAC clones were identified by the eight-copy restriction fragment length polymorphism (RFLP) marker, RG103, that cosegregates with *Xa21*. The three clones can be divided into two different nonoverlapping groups according to their Southern hybridization patterns: group 1 includes BAC 103-1 and BAC 103-5; group 2 includes BAC 103-7 (P.C.R., unpublished results). Most copies of RG103 hybridize to a 230-kb *Sfi* I DNA fragment by pulsed-field gel electrophoresis (PFGE) analysis (P.C.R., unpublished results). FISH analysis showed that all three clones hybridized to the same chromosomal region. Two-color FISH with BACs 103-1 and 103-7 confirmed their close linkage as their hybridization signals overlapped on both metaphase chromosomes and interphase nuclei (Fig. 1*f*). This result is in good agreement with PFGE analysis. The three BAC clones were physically mapped at the middle of the long arm of chromosome 11 (Fig. 1*g*) with a FL value of 0.50 (FL, fractional length, a fraction from the FISH signal to centromere over the total chromosome arm length). The chromosome identification is deduced from the position of RFLP marker RG103 in genetic linkage map 11 (23). The arm ratio (long arm/short arm) of chromosome 11 is 1.41 at somatic metaphase in NIL IR-BB21. However, chromosome 11 was identified as a metacentric chromosome by pachytene analysis in variety IR36 (24).

Two additional BAC clones, 818-13 and 818-14, were identified by DNA marker pTA818, originally derived from a random amplified polymorphic DNA band (RAPD818 in ref. 17). Clone 818-13 contains one copy of pTA818 that is monomorphic between the resistant and susceptible NILs. Clone 818-14 contains another copy of pTA818 that is polymorphic between the NILs and mapped to the *Xa21* locus on chromosome 11 (17). FISH analysis in NIL IR-BB21 showed that 818-13 has only one location. However, 818-14 has two different locations with one FISH site relatively small and the other relatively large in interphase nuclei. The two copies of 818-14 are clearly located on two different chromosomes by metaphase mapping (Fig. 1*h*). Two-color FISH showed that FISH signals from 818-13 overlapped with the smaller site of 818-14 and the larger site of 818-14 overlapped with clone 103-7 (Fig. 1*i*). Thus, 818-13 and the smaller copy of 818-14 are located on a different chromosome from 103-7 and are not linked to *Xa21*. In the regular rice line DV85, 818-14 has only one location and overlaps with the signals from 818-13 on both interphase and metaphase chromosomes (Fig. 1*j*). Cohybridization of probes 818-13, 818-14, and 103-7 indicates that clones 818-13 and 818-14 are located on a different chromosome from clone 103-7 in DV85 (Fig. 1*k*). Both 818-13 and 818-14 are located in a proximal region of a pair of unidentified chromosomes (Fig. 1*j*).

DISCUSSION

We have demonstrated that large genomic clones can be used for *in situ* hybridization mapping in rice, even though they may contain highly repeated DNA sequences. All BAC clones analyzed in the present study generated strong FISH signals, even with inserts as small as 40 kb. In a similar fashion, we have shown that cosmid clones can also be applied to FISH mapping of the rice genome (unpublished results). This technique provides an excellent approach to overcome the difficulty of *in situ* hybridization mapping using single- or low-copy sequences in plants. The successful application of this technique in other plant species will depend on both the size of the genomic clones analyzed and the percentage of repetitive DNA sequences in the genome. The rice genome contains a large percentage (≈75%) of single- or low-copy number DNA (25, 26) and this may contribute to the successful application of small genomic clones in FISH analysis. For other plant species, such as wheat (*Triticum aestivum* L.), with genomes containing >80% repetitive DNA sequences (27), relatively large clones will be needed to ensure that enough unique sequence will be available to generate distinctive signals.

FISH analysis provides a rapid method to determine the chimeric status of a genomic clone. A high percentage of chimeric clones in a genomic library, such as that observed in many YAC libraries, complicates map construction considerably. Thus, it is important to know the general status of chimerism in a library. Although chimerism can be analyzed by genetic analysis of the ends of the DNA insert in a specific clone, this approach is time consuming compared to data obtained by FISH. This is of particular importance in plant species with a high percentage of repetitive sequences, since most of the insert ends contain repetitive sequences that cannot be used for RFLP mapping or generating PCR probes for hybridization screening (28). The disadvantage of FISH analysis is that the FISH signals neither distinguish duplicated sequences from true chimerism nor discriminate between chimerism and cocloning or mixed colony propagation events. Nevertheless, comparative studies of FISH and molecular methods for determining chimerism in human YAC clones have demonstrated a high level of concordance (29). It is interesting to note that all clones from the rice BAC library derived from IR-BB21 (16) hybridized to a single location; similar results were obtained with clones isolated from a sorghum BAC library (28). This indicates that these libraries have a very low level of chimerism as compared to a rice YAC library in which 40% of the clones are chimeric (30).

Rice chromosomes are small and very difficult to identify individually on the basis of morphological differences. Reproducible banding techniques are not available for identification of the rice chromosomes. Only investigators with considerable experience and skill are able to identify different chromosomes by using well spread somatic prometaphase or meiotic pachytene preparations (24, 31). FISH analysis using genomic clones that contain genetically linked markers should provide a new technique for rice chromosome identification. BAC clones specific to each chromosome can be isolated from libraries and used as chromosome-specific FISH markers. It was shown previously that combinatorial labeling of probes with three different modified nucleotides permitted the detection of seven different probes simultaneously (5). Further development in this field should make it possible to visualize even more probes simultaneously (32). Therefore, it is possible in the future that all 12 pairs of rice chromosomes could be identified in a single multicolor FISH experiment with chromosome-specific FISH markers.

Chromosome-specific FISH markers will be valuable for aneuploid identification, such as trisomics, by interphase mapping. Well-spread metaphase or prometaphase cells, usually difficult to obtain, are not necessary in interphase mapping.

Chromosome-specific FISH markers also can be used to analyze other genetic stocks, such as translocation stocks. A series of markers located at different regions of a chromosome can be established and used to analyze specific translocation stocks, so that the breakpoint can be localized between two specific markers. Another possible application of chromosome-specific FISH markers is to analyze the structure of chromosomes from related species. For example, the gene synteny and homologous relationship of chromosomes from a wild rice species to a particular rice linkage group can be quickly analyzed by FISH mapping of these markers.

FISH mapping of the *Xa21*-related genomic clones demonstrated that this technique provides a valuable supplemental tool for map-based cloning in plants. This technique can be used for rapid determination of the chromosome location of a group of genomic clones. Chimeric clones or clones located on different chromosomes can be eliminated from the walking analysis. Characterization of the relationship among 818-13, 818-14, and 103-7 clones provided important information that would be difficult to obtain by conventional genetic and PFGE analysis. Based on the previous genetic data, the pTA818 sequence located in the 818-13 clone was monomorphic between the recurrent parent and NIL IR-BB21 and could not be mapped in the progeny (17). In addition, initial PFGE results from NIL IR-BB21 indicated that all DNA sequences in 818-13, 818-14, and 103-7 were located in the same chromosomal region (17). However, FISH analysis of these three clones quickly and clearly established that 818-14 is located very close to 103-7 but 818-13 is located on a different chromosome in NIL IR-BB21. Our recent PFGE data confirmed this result (P.C.R., unpublished data). The close genetic and physical linkage between 818-14 and 103-7 indicates that these clones provide a good starting point for chromosomal walking to the *Xa21* disease-resistance locus.

Interestingly, in both NIL IR-BB21 and DV85, a FISH signal from the 818-14 probe overlaps with the 818-13 signal. This result is likely due to the homology of a 1-kb pTA818 sequence shared by 818-13 and 818-14. It is not known how far the homology extends. In DV85, 818-14 maps to a single location and is therefore unlikely to be a chimeric clone. The two locations of 818-14 in NIL IR-BB21 could be explained as follows: in *O. longistaminata*, the pTA818 sequence may have transposed from an unlinked chromosome to the *Xa21* locus on chromosome 11, resulting in the observed sequence duplication in NIL IR-BB21. In support of this speculation, we recently discovered a high degree of similarity of the pTA818 sequence to a portion of the *Antirrhinum* gene *Tam1*, a member of the CACTA family of transposable elements (P.C.R., unpublished data).

An important future application of FISH mapping in plants is to determine the physical distance between genetically mapped clones. In a map-based cloning strategy, one of the most important steps is to estimate the physical distance separating two tightly linked markers flanking the target gene. Because of the uneven distribution of recombination along the physical length of plant chromosomes (33, 34), it could be difficult to determine such physical distance by conventional PFGE analysis. In these situations, FISH mapping can be applied to estimate the relative physical distance based on the separation distance of FISH spots in the interphase nuclei as demonstrated in humans (3). However, the relationship between separation distances of FISH signals in interphase nuclei and linear DNA distance in plant species remains to be established.

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