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Visual Detection of Useful Genes on Plant Chromosomes

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Abstract
In this manuscript the current status of direct visualization of gene location on chromosomes was reviewed by considering 5 aspects as follows: principle of in situ hybridization (ISH) method, historical perspective of mapping genes by in situ hybridization, improvement in the sensitivity of fluorescence in situ hybridization (FISH), visualization of location of useful genes on chromosomes, and prospects for visualization of useful genes.

Discipline: Experimental apparatus and method / Biotechnology / Plant breeding
Additional key words: physical mapping, rice, FISH

Principle of *in situ* hybridization (ISH) method

ISH is a method for the visualization of the location of nucleotide sequences on chromosomes, nuclei, and even in the tissues. The principle of the method involves the hybridization of labeled nucleotide sequences, or probes, directly to DNAs or RNAs with the complementary sequences in chromosomes, nuclei or tissues. The location of the labeled probes hybridized to the complementary DNA sequences in chromosomes, for example, is detected by fluorochrome-labeled antibodies that recognize the label of the probes under a fluorescence microscope. The ISH in which fluorescence is used to detect probes is referred to as "fluorescence *in situ* hybridization (FISH)".

Historical perspective of mapping genes by ISH in rice[2)]

In 1910, the rice chromosome number was determined to be 2n=24 by Kuwada[14)]. It took, however, more than 80 years until all the rice chromosomes were identified objectively and a rice chromosome map was developed by Fukui and Iijima[3)] using imaging methods[1)]. The long time interval from the determination of the rice chromosome number to the identification of individual chromosomes can be ascribed to the fact that rice has the smallest genome size of 430 Mb, thus the smallest chromosome size, among those of the main cereals.

Attempts to visualize specific DNA sequences directly on rice chromosomes have been pursued for years without the use of an objective method for identifying rice chromosomes nor a chromosome map at the beginning of the 1980s. Fukui et al.[6)] succeeded in physically locating 18S-5.8S-25S ribosomal RNA genes (45S rDNA) loci at the end of a pair of chromosomes with ^{125}iodine-labeled ribosomal RNA probes. This was the first reproducible result of *in situ* hybridization (ISH) using repetitive sequences in rice (Fig. 1a). The location of 45S rDNA loci in rice was detected by non-radioactive (biotin-labeled) probes by coloration using Japonica rice in 1991[8)] (Fig. 1b). FISH was successfully achieved in 1992 for the first time and variability in the number of 45S rDNA loci was revealed among the rice species[5)] (Fig. 1c). Multicolor FISH using different fluorescent colors simultaneously for detecting 5S rDNA and 45S rDNA was developed in 1994 soon after the successful development of FISH. Presently the multicolor FISH is a common method widely utilized to locate different nucle-

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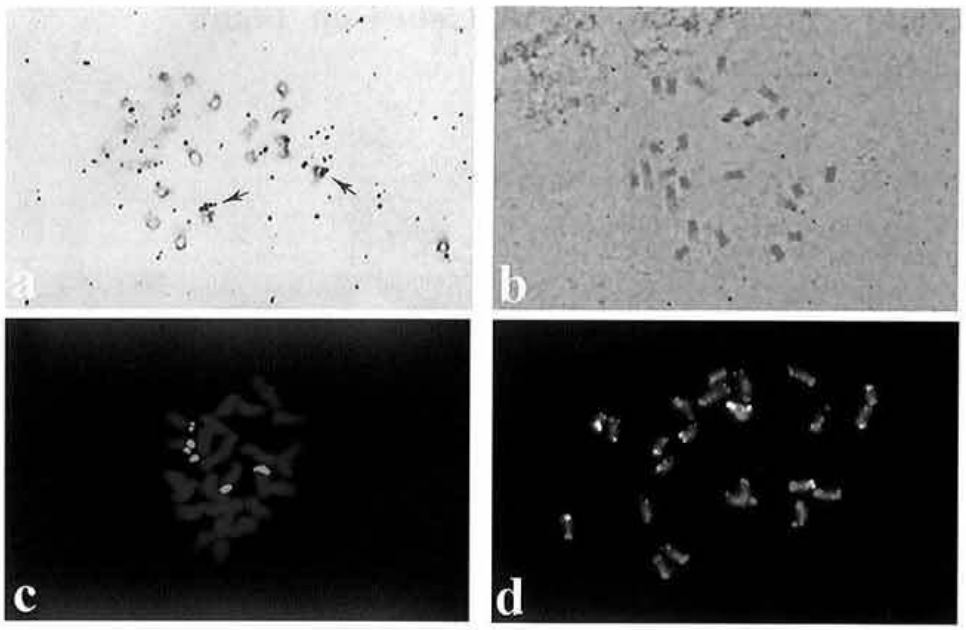

Fig. 1. Recent advances in *in situ* hybridization (ISH) for the past 15 years
a: Detection of ribosomal RNA gene (rDNA) locus with radioactive ribosomal RNA.
b: Detection of rDNA by a non-RI labeling method.
c: Detection of rDNA loci in Indica rice by FISH.
d: Detection of multi-color fluorescence from different probes (telomere, green and TrsA, red) simultaneously.

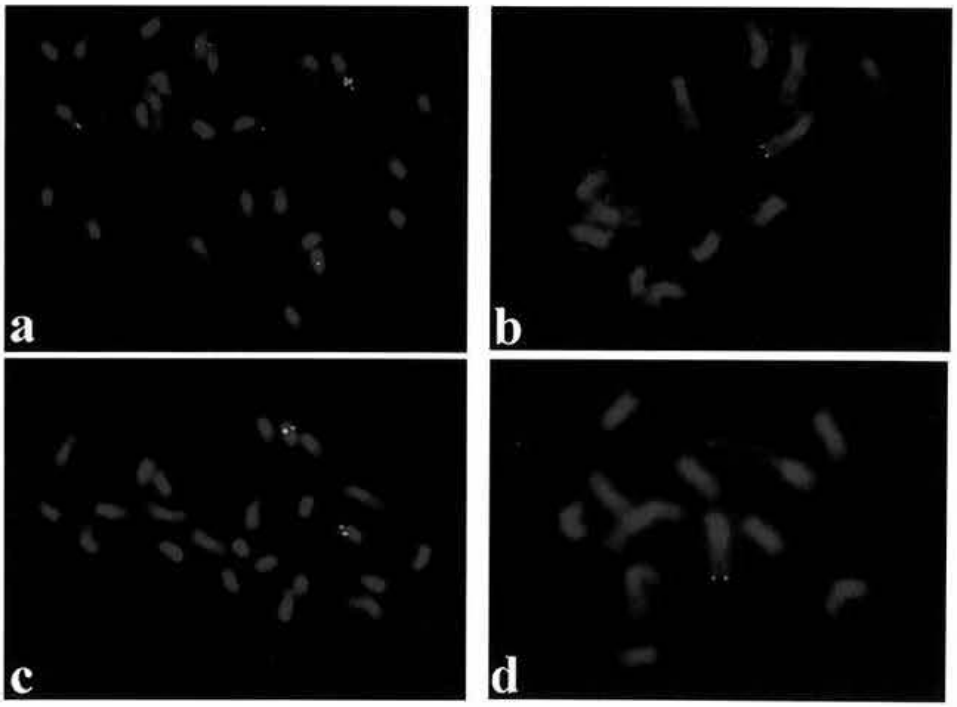

Fig. 3. Visualization of the location of useful genes on rice chromosomes
a: Gall midge resistance gene on chromosome 4.
b: Leaf blast resistance gene on chromosome 2.
c: Bacterial blight resistance gene on chromosome 11.
d: RFLP marker (*Xnp*247) on chromosome 4.

otide sequences simultaneously, such as telomere sequences (green fluorescence) and rice A genome specific tandem repeat sequence, TrsA (red fluorescence) (Fig. 1d)[18].

All these efforts have enabled the detection of highly repetitive sequences on rice chromosomes reproducibly and efficiently. The development of both the chromosome map and the FISH method has contributed significantly to the physical mapping of DNA sequences in rice.

Improvement in the sensitivity of FISH

Once reproducible detection of the repetitive sequences on rice chromosomes using the FISH method was obtained in the early 1990s, the main objective of FISH was to enhance the detection sensitivity of the fluorescent signals. Even though the visual detection of repetitive sequences on rice chromosomes had become routine work, detection of a unique sequence was considered to be difficult because of the smaller sizes of nucleotide sequences.

Fig. 2a shows clones differing in sizes, which were used as probes in the FISH experiments to evaluate the sensitivity of detection of fluorescent signals on rice chromosomes. In the clones with insert sizes ranging from 399 kb to around 1 kb, attempts were made to determine whether their fluorescence could be visualized on rice chromosomes after FISH[19]. The largest clones were those of yeast artificial chromosomes (YAC) with 399 and 340 kb of inserted rice genomic DNAs. A bacterial artificial chromosome (BAC) with a 180 kb insert, and a cosmid clone with a 35 kb insert were also tested using the FISH method. The smallest clones were molecular markers cloned into plasmid vectors with around 1 kb inserts.

To visualize these clones, both the experimental procedures and the detection equipment were improved. First, the detection sensitivity of faint fluorescent signals from the probe DNAs was enhanced by the introduction of a cooled CCD camera. The camera was directly mounted on top of a microscope. Second, in the FISH protocol, a longer hybridization period between the labeled probes and chromosomal DNAs was used. The longer hybridization period ensured hybridization between the probes and complementary DNA in the chromosomes. All the clones from nearly 400 to 1 kb were successfully visualized by the improved FISH method. As a result, the detection sensitivity of FISH was enhanced by 400 times throughout the experiments[19] (Fig. 2b).

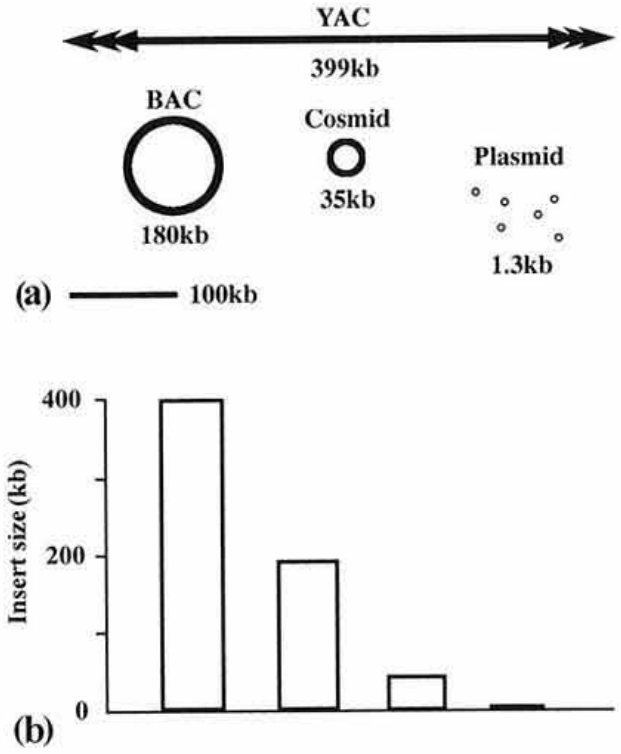

Fig. 2. Various sizes of probe DNAs used in FISH (a) and improvement of the detection sensitivity of FISH (b)

Visualization of location of useful genes on plant chromosomes

1) Mapping of a resistance gene to gall midge, Gm2

Fig. 3 shows the results of the improved FISH

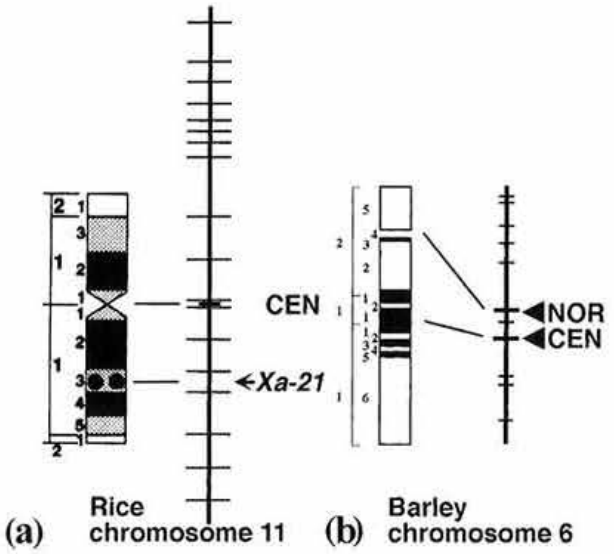

Fig. 4. Discrepancy between the chromosome and genetic maps for rice chromosome 11 (a) and barley chromosome 6 (b)

method using different DNA clones as probes. All the clones contained useful genes such as disease resistance genes. The result of FISH using a YAC clone (YAC5212) with a 340 kb rice genomic DNA was presented, because YAC5212 is flanking a gall midge (*Orseolia oryzae*) resistance gene, *Gm2*. Gall midge is a major dipteran insect pest of rice in India, China, Africa and Southeast Asia and its resistance genes are now being cloned. Fig. 3a shows the YAC signals on the pair of rice chromosome 4[21]. Fluorescent signals are detected in the interstitial region of long arms indicating the physical location of *Gm2*. The position of *Gm2* was also confirmed by the FISH using 2 RFLP markers genetically linked to *Gm2*.

2) Mapping of a resistance gene to leaf blast, Pi-b

The physical location of the resistance gene to rice leaf blast was then examined. Rice blast caused by *Pyricularia oryzae* (*Magnaporthe grisea* (Hebert) Barr) is the most important fungal disease of rice. The resistance gene, *Pi-b* was cloned into a BAC clone (BAC123) which has a 180 kb insert of rice genomic DNA. Because the location of the genomic DNA on rice chromosomes was unknown, the FISH method was employed to physically determine the actual location of the BAC clone or the position of the gene, *Pi-b*.

Fig. 3b shows the signals from the BAC clone at the end of the long arm of rice chromosome 2[17]. Haploid rice plant derived by anther culture was used for chromosome preparations. Thus one single-tagged chromosome shows clear doublet signals which are essential for the discrimination between genuine signals from noises. Thus the chromosomal location of the *Pi-b* gene was determined at 2p1.2 according to the rice chromosome map[2].

We further analyzed the BAC signals on the 10 chromatids of rice chromosome 2 by the imaging method to determine the precise location of the gene on the chromosomes. As a result, the *Pi-b* gene was located at the position of 96.16 ± 0.91 from the end of the short arm when the total chromosome length of chromosome 2 was 100.00[17]. This method was also employed to determine the 5S ribosomal RNA genes on rice chromosomes. The 5S rDNA locus was allocated to the position of 39.3 ± 2.6 on chromosome 11 (11p1.1) from the end of the short arm[10].

3) Mapping of a resistance gene to bacterial blight, Xa-21

The smaller rice genomic DNA of 35 kb nucleotide sequences cloned into a cosmid vector, was examined. The clone contained a resistance gene, *Xa-21* to the rice disease, bacterial leaf blight (*Xanthomonas oryzae*). Bacterial leaf blight is also a major rice disease widely distributed in Asian countries. The same improved FISH method was used to locate *Xa-21* on the rice chromosome. In this case, since we used the Indica rice variety IR36, a diploid plant material, the signals were observed in a pair of rice chromosomes.

Fig. 3c shows the fluorescent signals from a pair of rice chromosomes. By using the uneven condensation pattern, the rice chromosomes were identified as rice chromosome 11 and the doublet signals were clearly observed in the interstitial region on the long arm (11q1.3)[19].

4) Mapping of the molecular marker, Xpn *247*

Molecular markers are very useful for the construction of linkage maps and more than 2,000 molecular markers were developed to construct the rice linkage map[11]. The size of the molecular markers varies and the size of molecular markers, such as RFLP markers is often less than a few kilo basepairs. The RFLP markers played an important role in the construction of many linkage maps at the beginning of genome mapping. We applied the FISH method to locate a RFLP marker (*Xnp* 247) with 1.29 kb of rice genomic DNA.

Fig. 3d shows the signal position from the RFLP marker. The doublet signals were clearly detected at the end of rice chromosome 4 (4q2.1)[19]. The results indicate that even a nucleotide sequence with about 1 kb could be successfully detected by the improved FISH method. It also means that practically all the functional genes could be directly detected on the chromosomes by the improved FISH method[17]. Self-incompatibility-related genes of *Brassica* spp. have already been mapped by using FISH [9,11].

The physical mapping of the nucleotide sequences also reveals a discrepancy between the physical length of the chromosomes and the genetic distance calculated by the recombination values. Fig. 4 shows 2 examples of the discrepancy detected in rice[19] and barley[4]. Comparison of the chromosomal location of *Xa-21* determined by FISH and linkage analyses revealed a remarkable discrepancy between the total length in the 2 maps. The linkage map of chromosome 11 showed a much longer length when the chromosome map and the linkage map were aligned by the 2 landmarks i.e. the centromere and the position of *Xa-21* now determined[19] (Fig. 4a). As demonstrated in the barley chromosome map that was developed by imaging methods[4] and linkage map, the length of the satellite part, that is the terminal region of the chromosome 6 was markedly over-estimated in the linkage map. The discrepancy was again confirmed by a detailed comparison between the chromosome and dense

linkage maps in barley[12]. The over-estimation of the terminal length in the linkage map compared to the actual length of barley chromosomes determined by FISH and/or imaging methods suggests that recombination occurs frequently only in the terminal region of the barley chromosomes.

Although the discrepancy between the overall length of the chromosome and linkage maps was demonstrated in rice chromosome 11 (Fig. 4a), it remains to be determined whether the frequent recombination observed only in the terminal chromosomal regions could also occur in rice and other plant species as a general tendency.

Prospects for visualization of useful genes

Visualization or mapping of useful genes is important in genetic analyses. Since, not all the plants have as dense linkage maps as rice[13] and it is usually time-consuming and laborious to construct a dense linkage map, FISH is a useful and efficient alternative method in many plant species. FISH could be applied for the mapping of genes and clones regardless of plant species.

When molecular markers that sandwich a certain character can be found, the distance between the markers could be obtained by using FISH on chromosomes and extended DNA fibers (EDFs)[20]. Moreover, it may be possible to clone the gene by a FISH-oriented method and not by an ordinary map-based cloning method. First, FISH using the linked marker(s) could enable to determine the chromosomal position of the target gene. Then the laser dissection[7] or the ordinary dissection method could be applied to collect the chromosomal fragments with the signals. Finally, clones in the chromosomal regions could be obtained by the degenerated oligo-primer (DOP)-PCR method using the dissected chromosomal fragments as the templates. Direct amplification of ribosomal RNA genes[16] and α-amylase genes[15] by the laser-dissection method has been successfully applied in some cases.

In conclusion, the visualization of useful genes is important not only to determine their actual location on the chromosomes, but also it may be possible to clone the genes in conjunction with the chromosome dissection method even in plants with limited genome information. It is anticipated that the visualization method represented by FISH will be more useful when it is combined with new methods to manipulate chromosomes and even DNA fibers.

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