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Full Length Research Paper

Analyses of karyotypes and comparative physical locations of the resistance gene, *Xa-5*, between *Oryza sativa* and *Oryza officinalis*

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A rice BAC library is being used widely in rice genome researches due to its distinctive advantages over other library systems. Physical locations of the rice bacterial blight resistance gene, *Xa-5*, was mapped comparatively with a BAC clone linked to this gene, in *Oryza sativa*, a cultivated rice and *Oryza officinalis*, a wild rice. *Xa-5* was *in situ* hybridized to *O. sativa* and *O. officinalis* chromosomes. *Xa-5* was located on the short arm of chromosome 5 in *O. sativa* but on the long arm of chromosome 5 in *O. officinalis* with fraction length (FL) 48.85 and 47.30%, respectively and their FL were consistent with the results obtained by using the selective marker of rice, RG556, as a probe. The frequencies of signal detection of the marker, RG556 and the BAC clone, 44B4, were 8.0 and 41.3% in *O. sativa*, while 9.0 and 42.3% in *O. officinalis*, respectively. Based on a comparative RFLP map of a wild rice, *O. officinalis* and *O. sativa*, comparative analyses of karyotypes of *O. officinalis* were demonstrated firstly by fluorescent *in situ* hybridization (FISH) using a BAC clone and an RFLP marker from *O. sativa* as probes.

Key words: BAC-FISH, karyotype, *Oryza sativa*, *Oryza officinalis*, physical location, *Xa-5.*

INTRODUCTION

A wild rice is an important resource of new resistance genes obtained from the natural selective stresses of the complex geographic and ecological environment, which dictates more stable and wider spectrum of resistance than a cultivated rice (He, 1998; National Exploration Group of Wild Rice, 1984). As we know, there is gene-for-gene hypothesis between a resistance gene in cultivated rice and a non-pathogenic gene in pathogen, but the non-pathogenic genes often vary or new pathogenic genes appear. Therefore, it is necessary to introduce new resistance genes into rice cultivars continuously for maintaining and increasing the disease-resistance of the cultivated rice population. The wild rices provide main germplasmic resource pools of various resistance genes, so more and more research plans on wild rices are being put into practice (He, 1998; Sun et al., 2000; Lan et al., 2006a, 2006b; Dong et al., 2007).

There are three species of wild rices in China, *Oryza ruffipogon*, *Oryza meyeriana* and *Oryza officinalis*. *Oryza officinalis* is distributed over 38 counties in Guangdong, Guangxi, Hainan, Yunnan provinces (Gao et al., 2001; Lan et al., 2006b). It has been proven that *O. officinalis* in China has high resistance to many kinds of diseases and pests including blast, green leafhopper, tungro spherical virus, brown plant hopper and gall midge (National Exploration Group of Wild Rice, 1984). Therefore *O. officinalis* is an ideal material for resistance-breeding of rice. But the type of genome of *O. officinalis* (CC) is different from cultivated rice (AA) and the interspecific crossing often meets reproductive isolation and influences seriously the exchange of the genetic matter. Molecular marker analysis revealed that in southern South Asia the diploid CC genome species have a high level of genetic diversity compared to the diploid AA genome species (Nonnatus et al., 2006). The molecular technique has been used for identification of hybrids of wild rices, genetic component analyses of generations and physical locations and cloning of wild rice genes (Zhang et al., 1998).

A currently available rice genetic map has many

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molecular markers which are linked to resistance genes such as *Gm-2, Gm-6, Pi-5(t), Pi-2(t), Glh, Xa-5* and *Xa-21* (Causse et al., 1994; Gallego et al., 1998). The bacterial artificial chromosome (BAC) clones that include these genes have been screened with corresponding restriction fragment length polymorphism (RFLP) markers (Yang et al., 1997). *In situ* hybridization with these BAC clones are not only helpful to understand the structure of *O. officinalis* genome in detail, but also provide bases for isolation and utilization these resistance genes (Qin et al., 2000; Lan et al., 2006b).

In this manuscript the sites of *Xa-5* on chromosomes in *O. sativa* and *O. officinalis* with the rice RFLP marker, RG556 and the BAC clone, 44B4 that have been screened by using RG556 as probes based on a comparative genetic map between *O. sativa* and *O. officinalis* reported by Jena et al. (1994). At the same time, the mitotic karyotypes of chromosome 5 of *O. officinalis* were analysed comparatively by fluorescent *in situ* hybridization (FISH.).

MATERIALS AND METHODS

Preparation of C_0t -1 DNA

Rice C_0t -1 DNA was prepared as described by Zwick et al. (1997) and Lan et al. (2006b).

Plant material and chromosome preparation

O. officinalis Line 1589 was provided by Guangdong National Wild Rice Garden. P. R. China and *O. sativa* Guang Lu Ai 4 was obtained by Prof. Z.K. Zeng, Hubei Acadamy of Agriculture Sciences. Chromosomes were prepared according to the methods described by Yan et al. (1998) and Ren et al. (1997).

The rice BAC clones and probe labeling

The 137 kb BAC clone 44B4, provided by Dr. D.C. Yang at Wuhan University, was screened out by an RFLP marker RG556 that was obtained from Dr. S.R. McCouch at Cornell University (U.S.A.).

A BAC DNA and a plasmid DNA of RG556 were extracted by the standard alkaline lysis method (Sambrook et al., 1989). The purified probe DNA was biotin-labeled by nick–translation kit (Sino-America Biotechnology Company, Wuhan, China). The reaction mixture containing 0.5 µg probe DNA was incubated at 15°C for 1.5 - 3 h and terminated by adding 5 µl of 0.2 M EDTA (pH 8.0). The labeled probe was separated from un-incorporated nucleotides with the Sepharose CL-6B (Sigma, St. Louis, USA) column. Incorporation of biotin-11-dUTP was evaluated by means of dot blots followed by the straptavidin-alkaline phosphatase (BRL, Life Technologies, Wuhan China) detection.

In situ hybridization and detection

The protocol of *in situ* hybridization was modified slightly from that of Song et al. (1995) and Jiang et al. (1995). Fifty nanogram of the labeled BAC DNA was used for each slide and the hybridization mixture consisted of 50% deionized formamide, 8% dextran sulfate, 2 × SSC, 0.5 µg salmon sperm DNA and 2 µg rice C_0t -1 DNA (100 bp -1 kb). The hybridization solution was denatured for 10 min in boiling water bath at 100°C and allowed to reanneal with the C_0t -1 DNA in a ratio of 1:80 (probe: C_0t -1 DNA) for 1 h at 37°C to block the BAC repetitive DNA sequences.

The hybridization mixture was applied on a slide and incubated at 37°C overnight. The FISH signal was detected by three steps as follows: <1> The coverslip was removed and the slide was washed with 2 × SSC at 42°C for 5 min. Then, 10 µg/ml of FITC-avidin (Sigma) was added and incubated at 37°C for 30 min, followed by washing three times with PBS for 3 min. <2> Anti-avidin (Sigma) (5 µg/ml) was added and incubated at 37°C for 30 min, followed by washing three times with PBS for 3 min. <3> FITC-avidin (Sigma, St. Louis, USA) (10 µg/ml) was added and incubated at 37°C for 30 min, followed by washing three times with PBS for 3 min. All slides were stained with 50 µg/ml propidium iodide (PI). The *in situ* hybridization slides were examined and analyzed under an Olympus BX60 microscope. FISH photomicrographs were taken using Kodak ASA-400 color film.

RESULTS

Analyses of karyotypes in *O. officinalis*

A comparative RFLP map has been constructed in *O. officinalis* by using 139 genomic and cDNA probes that had been used previously to map RFLPs in *O. sativa* (Jena et al., 1994). Nine of the 12 chromosomes of *O. officinalis* are identified as being highly homologous to those of *O. sativa* and *O. officinalis* chromosome numbers are given in light of the distribution of *O. sativa* RFLP markers in *O. officinalis* (Jena et al., 1994). But the *O. officinalis* karyotypes have been analysed. Based on the study of Jena et al. (1994), the RG556 marker that came from chromosome 5 of *O. sativa* as well as the BAC clone 44B4 screened by using the RG556, respectively, were used as probes to define the karyotypes the chromosome 5 of *O. officinalis*. The average relative length (RL) of RG556 and 44B4, were 5.20 ± 0.05 and 5.22 ± 0.08 and the average arm ratio (AR) were 1.22 ± 0.04 and 1.24 ± 0.05, respectively. No satellite was observed. Therefore the types of chromosome 5 of *O. officinalis* was determined to be a metacentric (M) chromosome (Table 1, Figure 1).

Physical locations of probes linked to *Xa-5* in *O. sativa* and *O. officinalis*

The results of FISH with the RFLP marker RG556 and the BAC clone 44B4 in *O. sativa* and *O. officinalis* were shown in Tables 2 and 3 and Figure 1. The signals of RG556 and 44B4 which were linked to *Xa-5* were detected in the short arm of chromosome 5 in *O. sativa* but in the long arm in *O. officinalis*. The average distance from the signal spots of these two markers to centromere in *O. officinalis* were 45.86 and 47.30 (Table 2), while 47.35 and 48.85 in *O. sativa*, respectively (Table 3). The detection rates were 9.0 and 42.3% in *O. officinalis*, and 8.2 and 41.3% in *O. sativa*, respectively. These results

Table 1. Analyses of karyotypes of chromosome 5 in *O. officinalis.*

Probe	Relative length	Arm ratio	Chromosome number	Type of chromosome
RG556	5.20 ± 0.05	1.22 ± 0.04	5	M
44B4	5.22 ± 0.08	1.24 ± 0.05	5	M

All experiments were done three times and data are expressed as an average ± standard deviation.
M, Metacentric.

clearly demonstrated that the detection rates with BAC clones were much higher than those with RFLP markers (Tables 2 and 3) and a pair of signals was detected by BAC-FISH (Figure 1).

DISCUSSION

In 1994, Jena et al. made a comparative RFLP map between the cultivated rice, *O. sativa* (AA) and the wild rice, *O. officinalis* (CC). They numbered the *O. officinalis* chromosomes, which are the most homologous with cultivated rice chromosomes, in the same order of *O. sativa* chromosomes. For example, in *O. officinalis*, the chromosome which has more sequences homologous to the RFLP markers of chromosome 1 in a cultivated rice was named chromosome 1 in spite of its relative length and arm ratio. Results of Jena et al. (1994) indicated that the RFLP marker RG556 is located on chromosome 5 of *O. officinalis* and therefore, the RG556 was used as a marker for chromosome recognition. The relative length and the arm ratio of chromosome 5 detected by FISH with the RG556 were measured to analyze karyotypes based on the comparative genetic map of *O. sativa* and *O. officinalis* and to give numbers to the chromosomes which contain the same or similar genetic compositions. Therefore, it was shown that the comparative karyotypes are analysed in different species in light of sequences homology based on the comparative genetic map and these results will be helpful to understand further the development of chromosomes during evolution of different species. Recently, the karyotype of chromosome 4 in *O. officinalis* has been defined by BAC-FISH (Qin et al., 2000).

During last few years, plant (especially grass family) comparative genetic maps have shown the interrelationship between the genomes of some plants such as rice, maize, pearl millet, sorghum, sugar cane and oats (Gale and Devos, 1998; Devos and Gale, 2000). These efforts provide the good physical locations of important genes with FISH. In plants, *in situ* hybridization technique has been used mainly for mapping repetitive DNA sequences and multicopy gene families (Jiang and Gill, 1994; Lan et al., 2006b; Dong et al., 2007), but large number of cosmids, yeast artificial chromosomes (YACs), BACs and phage P1 derived artificial chromosomes (PACs) have been rapidly mapped and considered as being useful as probes since the current FISH technology has been greatly improved during last 10 years (Tibiletti et al., 1999). For example, BAC clones as probes would increase the annealling chance between a probe and a target sequence, because the average insert size of rice BAC clones is as large as about 100 kb (Shizuya et al., 1992). Therefore the BAC-FISH technique would greatly promote the signal detection rate of a single copy gene (Qin et al., 2000). The genes such as *Xa-21* (Jiang et al., 1995), rDNA of cotton (Hanson et al., 1995), *Glh* and RTSV (Yan et al., 1998), Pi-5(t) and Gm-6 (Qin et al., 2001) and *Gm-2* (Qin et al., 2002) were physically located on plant chromosomes successfully by BAC-FISH. In this study, the signal detection rates of 44B4 in *O. sativa* and *O. officinalis* were much higher than those of RG556 (Tables 2 and 3). Although the long insert of BAC clone 44B4, 137 kb, may contain many specific DNA sequences, the hybridization signals for each of the BAC clone used in this study were concentrated on only one chromosome region in *O. officinalis* (Figure 1). It implied that long DNA fragments in the BAC clones were never separated during the evolution process in which *O. sativa* and *O. officinalis* diverged and were syntenic between *O. sativa* and *O. officinalis*. Thus, the comparative BAC-FISH may not only provide the information about locations of individual genes or markers, but also demonstrate the synteny for a plenty of different single or low copy genes or markers among different species.

The BAC clone 44B4 was used as a probe to detect and locate the homologous sequences of *Xa-5* in *O. officinalis*. One site hybriding with 44B4 was detected on the long arm of chromosome 5 in *O. officinalis*, but on the short arm of chromosome 5 in *O. sativa*. These results suggested that the homologous sequences of *Xa-5* may be invert during the divergence of *O. sativa* and *O. officinalis*. When no C_0t *-1* DNA were used as a block, many singals were observed on chromosomes in *O. officinalis* (Figure 1D), which was the same as that in *O. sativa*. This implies that the moderate and high repeat seguences in *O. sativa* are present also in *O. officinalis*. Therefore, C_0t *-1* DNA is necessary when BAC clones from *O. sativa* were used as probes in *O. officinalis*.

At present, a comparative mapping between model plant species, of which the complete genome sequence is known and crop species has been suggested as a new strategy for the isolation of agronomically valuable genes (Mullet et al., 2002; Van Buuren et al., 2002). For example, a comparative mapping between a cultivated rice as a model plant and its relatives by BAC-FISH will further

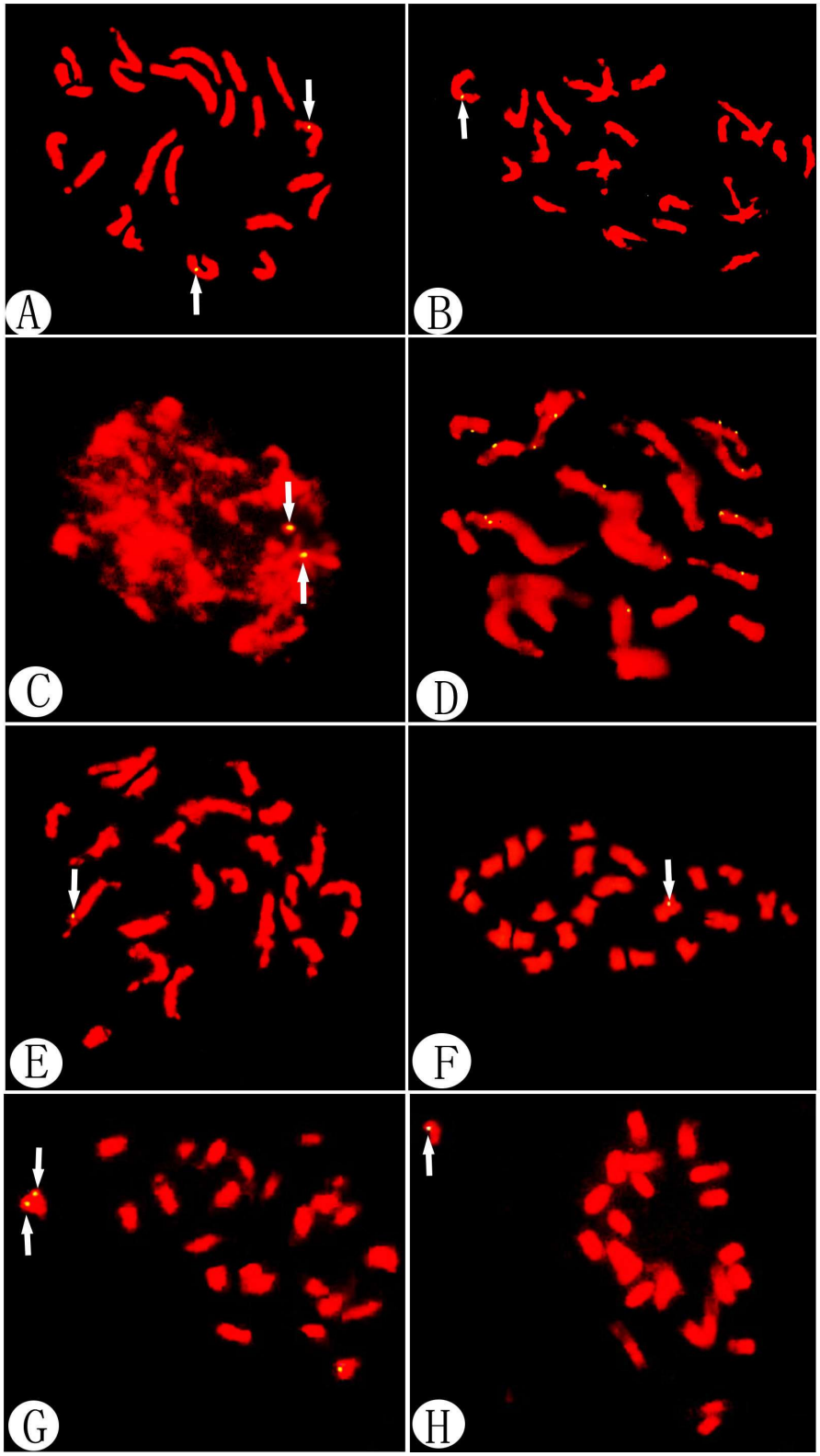

Figure 1. A and B. Signals of 44B4 on 5L in *O. officinalis*; C. Homologous signals of 44B4 in interphase chromatin in *O. officinalis*; D. Many signals of 44B4 observed on different chromosomes in *O. officinalis* when no C_0t *-1* DNA was used as a block; E and F. Signals of RG556 on the sister 5L in *O. officinalis*; G. Hybridization signals of 44B4 on 5S *in O.sativa*, two signals on the sister chromosome; H. Signal of RG556 on 5S *in O.sativa*.

Table 2. Locations of hybridization signals in *O. officinalis*.

Probe	Arm by detected signals	Arm ratio	Average distance from the signal spots to the centromere	Total number of cells examined	Number of the cells detected by signals	Detection rate (%)
44B4	5L	1.24 ± 0.05	47.30 ± 5.43	97	41	42.3
RG556	5L	1.22 ± 0.04	45.86 ± 4.67	134	12	9.0

All experiments were done three times and data are expressed as an average ± standard deviation.
L, Long arm.

Table 3. Locations of hybridization signals in *O. sativa.*

Probe	Aarm by detected signals	Arm ratio	Average distance from the signal spots to the centromere	Total number of the cells examined	Tumber of the cells detected by signals	Detection rate (%)
44B4	5S	1.73 ± 0.07	48.85 ± 3.40	145	60	41.3
RG556	5S	1.80 ± 0.04	47.35 ± 1.98	98	8	8.2

All experiments were done three times and data are expressed as an average ± standard deviation.
S, Short arm.

reveal their relationships and evolutionary history, realize common application of the research data, promote the exploitation and utilization of wild genetic resources and accelerate the establishment of the large genetic system for different species of *Oryza*, even for the genera of grass family.

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