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An Improved Method for Genomic In situ Hybridization in *Oryza* Species

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Abstract: For molecular characterization of F_1, hybrid of *O. sativa* × *O. officinalis* and its backcross-1 generation ($8C_1$) with *O. sativa* through genomic *in situ* hybridization (GISH), biotin labelled total genomic DNA from *O. officinalis* was used as probe. Cytological preparations were made by enzymatic maceration technique. Probe was hybridized onto chromosomal preparations at 37°C and signals were detected by colorimetric method using 3-amino-9-ethylcarbazole. Labelling efficiency of probe was determined by dot blot method prior to hybridization reaction. Based on the appearance of signal on chromosomes, it was inferred that there exists partial homoeology between the genomes of *O. sativa* and *O. officinal* and there are higher chances of gene(s) transfer from *O. officinalis* to *O. sativa.* More over the study shows that GISH is a powerful technique for genomic characterization of breeding material at any generation.

Key words: *Oryza sativa*, *O. officinalis*, *In situ* hybridization, Biotin labelling, GISH

Introduction

In situ hybridization (ISH) refers to the molecular cytogenetics technique which allows detection of specific nucleic acid sequences in morphologically preserved chromosomes, cells and/or tissues. Both RNA and DNA sequences can be labelled radioactively or non-radioactively and used as probe for molecular characterization of breeding materials including hybrids and advanced lines (Mujeeb-Kazi *et al*., 1996). It is a rapid tool to characterize chromosomes/chromatin material of hybrids (Schwarzacher *et al.,* 1989) to identify chromosorhal homology and homoeology (lqbal, 1998), chromosomal aberrations, genomic identifications (Mukai *et al.,* 1993), to construct physical maps of chromosomes and location of particular genes (Endo *et al.,* 1991). Since the first introduction of ISH using radioactive probes by Gall and Pardue (1969) and John *et al*. (1969), several modifications have been reported by different workers. Nonradioactive labelling in ISH started in 1982 with mapping of specific DNA sequences in chicken, *Drosophila* and mice (Singer and Ward, 1982; Langer-Safer *et al.*,1982; Manuelidis *et al.,* 1982). Rayburn and Gil (1986) reported the use biotin-labelled probes first time in plants for mapping 120-bp repetitive DNA sequences from rye on somatic metaphase chromosomes of common wheat. Since then several amendments have been reported by different workers depending upon the local conditions, cell/tissue type used and plant species under study. Le *et al.* (1989) used genomic *in situ* hybridization (GISH) to identify rye chromosomes in wheat-rye hybrid. With different ratios of labelled rye and unlabelled wheat blocking DNA, Mukai *et al.* (1992) discriminated rye chromosomes from that of wheat chromosomes in wheat-rye amphiploid through GISH. Leitch *et al.* (1990) used cell spread and tissue sections of the F_1 hybrid of *Hordeum chilense* × *Secale africanum* to characterize the parental genomes through GISH. This technique was also successfully used in wheat (Schwarzacher *et al.,* 1992; Mukai *et al.,* 1993; Mujeeb-Kazi *et al.,* 1996), *Avena sativa* L. (Chen and Armstrong, 1994), *Milium rnontianum* (Bennett *et al*., 1992), *Brassica campestris* (Iwano *et al.,* 1998) and *Oryza minute* and *O. latifolla* (Fukui *et al.,* 1997) for molecular cytogenatice of hybrids and advanced materials.

In present study GISH was used to characterize parental genomes in derivatives of a hybrid between *O. saliva* and *O. officinalis* to explore the possibilities of transferring useful gene(s) like genes for resistance to brown planthopper, white backed planthopper, bacterial blight, tungro, etc. from *O. officinalis* to *O. sativa.*

Materials and Methods

Plant materials: Plant material used in this experiment comprised *O. sativa* L. (an elite breeding line, 1 R65600-81-5-3 2 of new plant type (NPT)), *O. officinalis* Wall Ex Watt. (accession 100896), F_1 hybrid of *O. sativa and O. officinalis* and its progenies backcrossed with *O. sativa* (BC_1).

DNA isolation: Healthy and clean leaves from field grown plants of *O. officinal* and were collected in plastic bags and were kept on ice. Approximately 5-10 grams of fresh leaf samples were cut into small pieces and ground in liquid nitrogen using mortar and pestle. After initial processing of the plant tissue, DNA extraction was carried out following potassium acetate method (Dellaporta *et al.,* 1983) with minor modification.

DNA quality and quantity: To check the quality of isolated DNA, 3-4 of test DNA was mixed with 5-8 *Ail* of 5× tracking dye (bromophenol blue + xylene cyanol FF). The DNA was loaded onto 0.8% agarose in I× TAE buffer (0.02 M Trizma base, 0.57 ml glacial acetic acid, 1.0 ml of 0.5 M EDTA and adjusted volume to 500 ml with distilled water) containing 0.25-0.5 1.49/ml of ethidium bromide. The same volume of control DNA of known concentration was also loaded along with lambda DNA digested with Hindlll restriction enzyme to serve as molecular weight marker. Gel was electrophoressed between 50-60 volts for 45 min and visualized under UV light. High molecular weight DNA without any contamination of RNA was considered to be of good quality.

Quantity of the isolated DNA was checked by digesting equal volumes of test and control DNAs with *EcoRI* at 37°C overnight. Digested DNAs were run on agarose gel with molecular weight marker as described earlier. For accurate estimation of DNA concentration, lanes containing test DNA were compared with the lanes containing known concentration of DNA and molecular weight marker.

DNA labelling and efficiency testing: Total genomic DNA from *O. officinalis* was labelled with biotin-14-dATP by nick-translation system (Bethesda Research Laboratories, BRL) under supplier's instructions. Labelling was carried out at 15°C

for 90 min. To determine the efficiency of labelling, dot blot procedure was used. The important steps are given below: Four concentrations of DNA (50, 5.0, 0.5 and 0.05 ng/41) were blotted onto a piece of nitrocellulose membrane (Hybond N + Amersham). The membrane was dried at room temperature and baked at 80°C for 60 min. The baked membrane was transferred to vinyl bag (hybridization bag) containing 3% BSA (Bovine Serum Albumin) solution in buffer 1 (0.1 M Tris-HCI pH7.5, 1 M NaCl, 2 mM $MgCl_2$, 0.05% Triton X-100) and incubated for 20 min at 42°C with occasional shaking. It was transferred to a new vinyl bag containing 0.2% avidin alkaline phosphates° (API solution in buffer 1 and incubated for 10 min at room temperature. The membrane was washed three times at room temperature for 15 min each with buffer 1 followed by another series of three washings with buffer 2 (0.1 M Tris-HCI p1-19.5, 1 M NaCl, 5 mM $MgCl_2$) for 10 min each at room temperature. Both series of washings were coupled with occasional shaking. The membrane was then transferred to a new vinyl bag containing NBT and BCIP (10 each) solution mixed with buffer 3 (0.1 M Tris-HCI pH9.5, 0.1 M NaCl, 5 mM $MgCl_2$), sealed, covered with aluminum foil and incubated at room temperature for 60 min. Color reaction was stopped by washing membrane with distilled water briefly. Membrane was dried in dark and photographed.

Somatic chromosome preparation: Slides for mitotic chromosomes were prepared from root tips collected from field grown plants. Excised roots (1-2 cm) were pretreated with 2 mM 8-hydroxyquinoline for 30 min and fixed into a fixative (absolute ethanol mixed with glacial acetic acid at a ratio of 3:1) for 24 hours at room temperature. After fixation, root were washed thoroughly with distilled water followed by washing for 3-5 min in citrate buffer, pH 4.6 (0.01 M each of citric acid monohydrate, $C_6F1_80_7.H_20$ and tri-sodium citrate dihydrate, $C_01\text{-}1_50_7Na_3.2H_20$). Root tips (1-2 mm) were subjected to enzymatic maceration (3% cellulase "Onozuka" R10, Yakult, Tokyo, Japan + pectolyase Y-23, Seishin Pharmaceutical, Tokyo, Japan in citrate buffer) in a watch glass at 37°C for 60-90 min. The roots were thoroughly washed first in citrate buffer and then in distilled water to remove the enzyme solution. After washing in water for 5-10 min, root tips were squashed on clean slides using a few drops of fixative. Slides were air dried and stored in desiccator at room temperature.

Genomic *in situ* hybridization (GISH): Air dried slides were dehydrated by passing through ethanol series (70, 80, 95 and 100% ethanol, 5 min each) at room temperature. Hybridization mixture consisted of 50% deionized formamide, 2×SSC (standard saline citrate; 0.3 M NaCl, 0.03 M tri-sodium citrate dihydrate, pH7.0), 10% dextran sulfate, 2-3 µl labelled probe (50 ng/µl). The hybridization mixture was denatured for 10 min at 80-100°C and then immediately quenched on crushed ice for 5-10 min. Denatured probe mixture (40-50 Ml) was applied to each pre-dehydrated slide and covered with cover glass. The chromosomal and probe DNAs were denatured again simultaneously in an incubator at 75-80°C after placing slides in a humidified chamber for 10 min and transferred immediately to 37°C. The slides were left for hybridization for 15-20 hrs.

Detection of hybridization signal was done by colorimetric method. After hybridization cover glasses were removed by dipping the slides in 2x SSC followed by rinsing in 2x SSC at room temperature for 5 min, 2x SSC at 37°C for 10 min, 2x SSC and Ix PBS (phosphate buffer saline (0.13 M sodium chloride, 0.007 M sodium phosphate dibasic, 0.003 M sodium phosphate monobasic, pH7.4)1 at room temperature for 5 min each. Excess of PBS was drained off. The slides were then incubated at 37°C for 60 min in humidity chamber with 500 glislide of Detek-hrp detection reagent 10.01 M Phosphate buffer, 0.15M NaCl, 0.3% gelatin, 0.025% Triton X-100, 5 del Detek-hrp complex, ENZO Biochemical Ltd.]. After washing with 1x PBS for 5 min at room temperature, slides were drained off and were incubated at 37°C for 30 min in dark alongwith 500 Al of color reaction mixture (125 Ml 8x reaction buffer, 1 3-amino-9-ethylcarbazole, 875 *Al* distilled water) per slide. The slides were rinsed in Ix PBS to stop the color reaction and stained with 2% Giemsa stain for 1-2 min. Slides were then air dried and examined under the microscope to detect hybridization signals on the chromosomes using coloured filters. Photomicrographs were taken on Kodak color ASA 160 film using Zeiss Axiophot microscope.

Results and Discussions

Efficiency of labelling: Different concentrations of the biotin labelled probing DNA blotted on to a nitrocellulose membrane were detected with Streptavidin-AP-conjugated antibodies (BRL). The results of dot blot hybridization to determine the efficiency of labelling and to select the appropriate concentration of the labelled total genomic DNA to be used as probe are shown in Fig. 1. The hybridization signal appeared weaker with decreasing concentration of blotted. After different trials, 2-3 µl of 500 µl pl concentration per slide of probe was found to be satisfactory for GISH experiments to discriminate the parental genomes in F_1 and BC_1 derivatives. Dot blot hybridization method has been used by many workers in different species to identify the labelling efficiency of total genomic DNA used as probe and labelled with different heptans like biotin, digoxigenin and to monitor the strength of labelled probes to discriminate the DNA of species used in various experiments. For example, Schwarzacher *et al*. (1989) used dot blot DNA hybridization method to see the DNA similarities between *Secale africanum, Hordeum chilense* and *H. vulgare* using biotinylated genomic DNA of *S. africanum* as probe. They concluded that two species of *Hordeum* share little sequence homoeology to the *Secale* genomic DNA. Parokonny *et al.* (1992) monitored the efficiency of biotinylated total genomic DNA from *Nicotiana sylvestris* to discriminate among *N. sylvestris, N. plumbaginifolia* and *Atropa belladonna* using dot blot hybridization. Labelling success of DNA probes with biotin as well as digoxigenin reporters can also be monitored by dot blot hybridization procedure using Streptavidin-AP-conjugates and antidigoxigenin-AP-conjugated antibodies respectively for detection (Scherthan *et al.,* 1992). Thus the dot blot method is a time saving technique for probe testing prior to use it in *in situ* hybridization experiments.

Genomic *in situ* hybridization (GISH): *In situ* hybridization experiments were carried out on well spread somatic chromosomal preparations. The enzymatic maceration protocol for chromosome preparation was proved quick and reliable method to have well spread and clean chromosome slides suitable for GISH experiments. Some representative cells showing somatic chromosomes prepared by this method and used in *in situ* hybridization experiments are shown in Fig. 2. Total genomic DNA from *O. officinalis* was labelled with biotin and hybridized with metaphase chromosomes of the F_1 hybrid. The hybridization signal appeared on 12 chromosomes of *O. officinalis* as dark brown color while the other 12 chromosomes from *O. sativa* appeared as light blue due to Giemsa counter-staining (Fig. 3A). In addition to metaphase chromosomes, hybridization of the probe was also carried out on interphase cells with dark brown hybridization signal on the chromatin material (Fig. 3B). In some cases, the hybridization signal was also observed on few chromosomes of *O. sativa.* Of

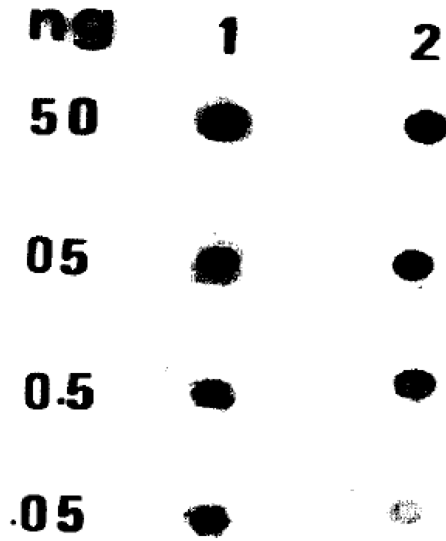

Fig. 1: Dot blot hybridization showing intensityof signal using different concentrations of biotin labelled DNA of *aofflc/nrilis* on the nitrocellulose membrane with DNA samples 1 and 2

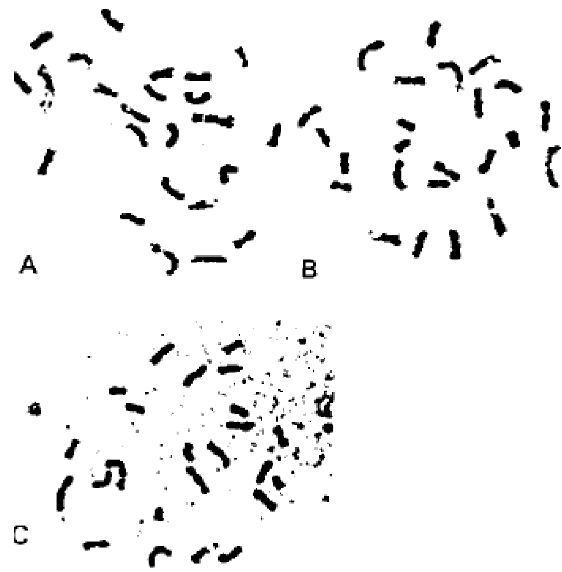

Fig. 2: Representative cells showing somatic metaphase chromosomes of the two parents and Fthybrid of *O. Baths* x a °*Moine/is* used in *in situ* hybridization; *A: O. seam* (1R65600-81-5-3-21, 2n = 2x = 24; B: a *officinal* and (accession 100896), 2n = 2x = 24; C: Fi hybrid ia *sativa* × *O. officinal/*1A showing 2n = 2x = 24 chromosomes

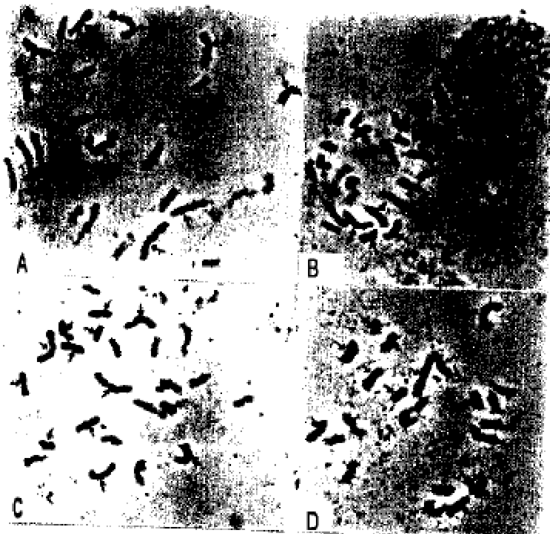

Fig. 3: Somatic metaphase chromosomes of Fi hybrid of *O. sativa O. affidavits* after genomic *in situ* hybridization using biotin labelled DNA of 0. officinally as probs. A: A cell showing hybridization signal on 12 choromosomes of *O. Officialls* (arrows). B: A cell showing hybridization signal on 12 chromosomes (arrows) along with and Interphase call (arrow head) showing dark brown hybridization signal on chromatin; C: A cell with hybridization signal on 13 chromosome (1 2 of *O. officinalis* +1 of *O. sativa*); D: A cell with hybridization signal on 15 chromosomes (arrows), (12 of *O. officinal*+ 3 of *O. sativa*)

Fig. 4: GISH on somatic metaphase chromosomes in $8C_1$ (*O. sativa* x *O. officinalis*/*O. sativa* 2n=3x =36. The biotinylated total genomic DNA of *O. officinalis* was used as probe; A: Dark blue hybridization signal (arrows) appeared on 12 chromosomes of *O. officinalis* only while 24 chromosomes of *O. sativa* appeared as light blue; B: Hybridization signal appeared on 16 chromosomes (12 of *O. officinalis* + 3 of *O. sativa*) indicated by arrows, while 21 chromosomes of *O. sativa* were light blue (unlabled)

the 70 cells of the F_1 hybrid observed after GISH, 8 cells (11.43%) showed hybridization signal on more than 12 chromosomes. Figs. 3C and D show the signal present on 13 chromosome (12 *officinalis* + 1 *sativa)* and 15 chromosomes (12 *officinalis* + 3 *sativa*) respectively.

In characterization of BC_1 14 *sativa* x *O. Officinalis/0 sativa),* total genomic DNA of *O. officinalis* labelled with biotin-14-dATP was used as probe to characterize parental genomes and to identify restructured chromosomes if any in said BC,. The dark blue hybridization signal appeared mostly on 12 chromosomes while unlabelled 24 chromosomes of *O. salve* were seen as light blue due to counter-staining with Giemsa (Fig. 4A). However, in some cells, the hybridization signals appeared on more than 12 chromosomes. Fig 4B shows hybridization signal on 15 chromosomes (12 *officinalis* + 3 *sativa*) in BC.. Of 30 cells *observed,* only 2 cells (8.67%) showed signals on more than 12 chromosomes while in other 28 cells (93.33%), hybridization signal was seen only on 12 chromosome of *officinalis.* The signal on extra chromosomes indicated that these chromosomes are either of restructured nature or possess homoeologous relationships with *O. sativa* gename as was seen in F_1 hybrid of the parents.

Quite a few workers used GISH for characterization of parental chromosomes in *Oryza* species. Fukui *et al.* (1997) identified 24 D-genome chromosomes out of 48 chromosomes of allotetraploid *O. latifolia* (CCDD). B-genome chromosomes were also discriminated from C-genome in *O. minute* (BBCC) using *O. officinalis* (CC) total genomic DNA as a probe in GISH experiments. Abbasi *et al.* (1998a) identified 14 chromosomes of *O. australiensis* and 13 of *O. sativa* in an anther culture derived plant from *O. sativa* x *O. australiensis* with 27 chromosomes in somatic cell and also identified 12 chromosomes of *O. brachyantha* in F_1 hybrid of *O. sativa x O. brachyantha* using Abbasi *et al*.(1998b). To best of our knowledge, there is no report to identify genomes in F_1 hybrid of *O. sativa* x *O. officinalis* using *in situ* hybridization except Asghar *et al.* (1998) when they discriminated 12 chromosomes of *O. officinalis* from that of other 12 chromosomes of *O. sativa* in the F_1 hybrid of *O. sativa* x *O. officinalis* using genomic DNA of *O. officinalis* as probe with fluorochrome mediated detection. The results show that *in situ* hybridization is a useful and time saving technique for genomic identification in any generation of the breeding materials. It further confirms that there exists partial homoeology between "A" and "C" genomes of *O. sativa* and *O. officinalis* respectively and blocking of these homoeologous DNA sequences are required to clearly discriminate the two genomes in cytological preparation using GISH. It means there are higher chances of transfer of economically useful gene(s) from *O. officinalis* to the cultivated rice.

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