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Full Length Research Paper

Genomic affinity between *Oryza sativa* and *Oryza brachyantha* as revealed by *in situ* hybridization and chromosome pairing

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Genomic affinity between *Oryza sativa* (2n = 24 AA), and *Oryza brachyantha* (2n = 24 FF) was assessed by using three strategies: genomic *in situ* hybridization (GISH), meiotic chromosome pairing, pollen and spikelet sterility. The chromosome pairing was examined in pollen mother cells of *O. brachyantha*, *O. sativa* and the hybrid between *O. sativa* and *O. brachyantha*. The hybrid was highly sterile with no pollen stain ability. Both parents showed regular meiosis with normal chromosome pairing. The F1 hybrid exhibited limited chromosome pairing. On an average, 0-2 bivalents and 20-24 univalents were recorded at metaphase-1 and 0 - 1 univalent at diakinesis. The most frequent configuration was two bivalents and twenty univalent. The meiosis was highly irregular showing unequal distribution of chromosomes at anaphase, formation of multipolar bodies and variation in the cell cycle of both genomes. GISH revealed unequivocal discrimination of *O. brachyantha* chromosomes as appeared red from *O. sativa* chromosomes that fluoresced yellow. No cross hybridization was examined between the labeled genomic DNA of *O. brachyantha* and the chromosomes of *O. sativa*. Mitotic chromosomes of *O. brachyantha* and *O. sativa*, in the hybrid, were discriminated by GISH. High sterility in this hybrid could be due to abnormal meiosis and lack of pairing.

Key words: Genomic *in situ* hybridization, wide hybrid, chromosome pairing.

INTRODUCTION

Wide hybridization is one of the plant breeding approaches for incorporating alien genetic variation to cultivated species. The first step in incorporating desirable alien chromosome segment is to produce the hybrid between wild and cultivated species and to establish alien addition lines and introgression lines. Hybrids between rice are distantly related wild species, usually difficult to produce. These hybrids are completely male sterile. Subsequent backcrosses are made with the recurrent rice parent to produce disomic progenies (2n = 24). Embryo rescue is used to produce F1 and backcross progenies until fertile plants with normal diploid chromosome complement (2n = 24) or 2n = 25 (monosomic alien addition lines) become available. The fertile progenies are selfed to produce advanced introgression lines and evaluated for transfer of useful traits (Brar and Khush, 1997). At the International Rice Research Institute (IRRI), a series of hybrids and monosomic alien addition lines have been produced through embryo rescue following hybridization between elite breeding lines of rice and several distantly related species of *Oryza* (Abbasi et al., 2009).

Many important characteristics have been incorporated into rice by alien gene transfer that is grassy stunt resistance from *Oryza nivara* (Khush, 1977), bacterial blight (BB) resistance (*Xa*21) from *Oryza longistminata* (Khush et al., 1990), brown plant hopper (BPH) and white backed plant hopper (WBPH) resistance from *Oryza officinalis* (Jena and Khush, 1990), blast and bacterial blight resistance from *Oryza minuta* (Amante et al., 1992), cytoplasmic male sterility from *Oryza perennis* (Dalmacio

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Abbreviations: GISH, Genomic *in situ* hybridization; **BPH,** brown plant hopper; **WBPH,** white backed plant hopper; **BB,** bacterial blight; **SSC,** standard saline citrate; **SSS,** sonicated salmon sperm; **BSA,** bovine serum albumin; **PMCs,** pollen mother cells.

et al., 1995) and bacterial blight resistance from *Oryza brachyantha* (Brar et al., 1996). *O. brachyantha* (2n = 24) is a diploid species having FF genome. It is resistant to BB and blast and yellow stem borer. It is distributed in Africa. Meiosis in wide hybrid is known to be highly irregular. This irregularity is the main reason for sterility. Pairing failure within hybrid may be due to insufficient homology between different parental genomes.

Variations in morphological traits, geographical distribution, cytogenetic relationship, breeding systems, cross-compatibility and biochemical markers have been used extensively to elucidate the relationships among species (Aggarwal et al*.,* 1997, 1999). The information on the assessment of genomic relationship between these two species by multiple approaches was lacking. In the present investigation we were able to establish genomic relationship between the chromosomes of these two species.

MATERIALS AND METHODS

Plant materials comprising of *Oryza sativa* (IR56), *O. brachyantha* (Acc.101232) and the hybrid between IR56 and *O. brachyantha* were kindly provided by the International Rice Research Institute (IRRI).

Preparation of genomic DNA

The genomic DNA was isolated from 5-10 g fresh leaves from *O. brachyantha* and *O. sativa,* using the method of Dellaporta et al. (1983). The DNA was digested with EcoR1 and labeled with Biotin-14-dATP, by nick translation (Gibco BRL), according to standard nick translation labeling system.

Genomic *in situ* hybridization

The hybridization mixture, containing 120 ng of biotinylated probe, 50% formamide, 3 µg sonicated salmon sperm (SSS) DNA, 2× standard saline citrate (SSC) and 2.4 µg unlabeled *O. sativa* DNA was denatured at 80°C for 10 minutes and immediately quenched in ice for 5 min. An aliquot of 18 µl was dropped on each slide, covered with cover slip, sealed with paper bond and air dried. The chromosomes denatured at 80°C for 10 min, using thermal cycler (Hybaid), followed by incubation at 37°C for 18 h. The cover slips were removed in 2xSSC, and the slides were washed with 2xSSC two times, and once with 4xSSC at 42°C for 10 min each. An aliquot of 100 µl of blocking solution, containing 5% bovine serum albumin (BSA) in 4×SSct (4×SSC + 0.05% Tween-20), was dropped on each slide, covered with cover slip and incubated for 5 min at 37°C. An aliquot of 70 µl fluorescein isothiocynate (FITC)-Avidin (Boehringer Mannheim) in 1% BSA/4xSSct was layered on the slides and incubated for 60 min at 37°C. The slides were washed three times with BT buffer (Sodium carbonate + tween 20) for 10 min each at 37°C. After washing, the blocking was carried out by 5% (v/v) goat serum (Cosmo Bio. Ltd.) for 5 min at 37°C. An aliquot of 70 µl biotinylated-anti-avidin solution in 1% BSA was dropped on each slide, and incubated for 60 min at 37°C. The slides were washed thoroughly with BT buffer twice and once with 2×SSC for 10 min each at 37°C, dehydrated in ethanol series: 70, 95 and 100% for three min each at room temperature.

The chromosomes were counterstained with propidium iodide (PI) Sigma), 1 µg/ml in water for 2 min. Each slide was mounted with 15 µl of vectashield. The slides were screened with fluorescence microscope (Axiophot Zeiss), equipped with filte set no. 05, 09 and 25. Photographs were taken with Kodak Ektacolor, ASA/ISO 400.

Meiotic chromosome preparation

Panicles were collected from the field grown plants of *O. sativa*, *O. brachyantha*, and their F1 hybrid (*O. sativa* × *O. brachyantha*). These were fixed in acetic alcohol (3:1) for 24 h. Anther at suitable stage were squashed in 2% aceto-carmine under cover slip and examined for chromosome association.

Mitotic chromosome preparation

Newly emerged roots (1-2 cm) from filed grown F1 hybrid plants were excised and treated at room temperature with 5mM 8-hydroxyquoniline (Sigma) for 30 min. The roots were washed thoroughly with distilled water and fixed in ethanol/glacial acetic acid (3:1) for 24 h at room temperature. To prepare chromosome squashes, the roots were taken out of fixative and thoroughly washed with distilled water and citrate buffer (0.01 M Citric acid monohydrate + 0.01 M Trisodium citrate dihydrate, pH 4.6). Meristematic portion of root tips were subjected to enzymatic maceration, 3% cellulose (Onozuka R10) + 2% pectolyase (Y-23) at 37°C for 1 h. After enzyme treatment, roots were again thoroughly washed in citrate buffer and distilled water. The cells were spread on the slide in a drop of fixative (3 parts of 95% ethanol + 1 part of acetic acid). The slides were air dried and used for *in situ* hybridization.

Pollen sterility

Pollen sterility was determined from 500 pollen grains from parents and F1 hybrid. The spikelets were collected near anthesis in I_2KI solution. Darkly stained and round pollen grains were counted as fertile while lightly stained and shriveled were counted as sterile. Pollen sterility was determined as follows: pollen sterility (%) = (no. of sterile pollen grains / total no. of pollen grains) x 100.

RESULTS AND DISCUSSION

We used three strategies to assess genomic affinity between *O. brachyantha* and *O. sativa*: meiotic chromosome pairing in the hybrid, pollen sterility and the level of cross hybridization of genomic DNA of one species with that of the chromosomes of other species. Genomic *in situ* hybridization (GISH) is a powerful technique for assessing the genomic relationship among species (Abbasi et al., 2010). Differential painting of chromosomes by GISH was used to look into the genomes of F1 hybrid (*O. sativa* × *O. brachyantha*). The *O. sativa* DNA when used as labeled probe to the chromosomes of hybrid as shown in Figure1, the probe preferentially hybridized to *O. sativa* chromosomes that fluoresced green due to fluorescein isothiocyanate (FITC) under blue light excitation. The probe produced uniform labeling pattern on all the *O. sativa* chromosomes allowing the identification of parental chromosomes of both species.

Both genomes discriminated without using blocking DNA and there was no cross hybridization between probes

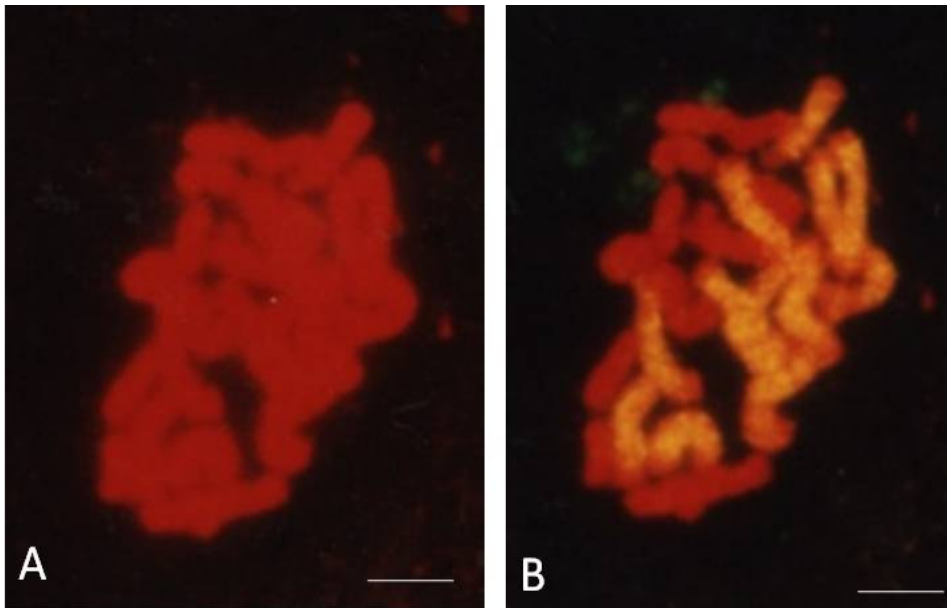

Figure 1. Genomic *in situ* hybridization using labeled *O. sativa* DNA as probe on Somatic metaphase chromosomes of the hybrid *O. sativa* x *O. brachyantha.* A) Metaphase cell stained with propidium iodide (PI) that fluoresced red; B) same cell hybridized with labeled genomic DNA from *O. sativa* and counterstained with PI. The labeled *O. sativa* chromosomes fluoresced yellow and unlabeled *O. brachyantha* chromosomes appeared red.

DNA and the chromosome of *O. brachyantha*. This indicates that the sequences are not similar between the genomes of *O. sativa* and *O. brachyantha*. The *O. brachyantha* chromatin was distinguishable at all the stages of the cell cycle. In interphase nuclei, *O. brachyantha* chromatin appeared as distinguished domain and did not intermix with *O. sativa* chromatin. GISH analysis support the earlier conclusion drawn, based on meiotic feature of this hybrid that the two genomes A and F are distinct (Brar and Khush, 1997). Lee et al. (2005) established the genomic relationship among the species on the basis of cento repeat and concluded that A and F genomes are highly divergent.

Meiotic chromosome behavior of *O. sativa* cv IR 56. *O. brachyantha* and the F1 hybrid between *O. sativa* x *O. brchyantha* was studied at pachytene, diakinesis, metaphase 1 and anaphase 1. *O. sativa* and *O. brachyantha* showed regular meiosis with normal chromosome pair forming 12 bivalents at diakinesis and metaphase 1. The bivalents were of rod and ring shaped. Ninety five percent of the cells showed normal distribution of chromosome (12/12) at anaphase 1. In our studies, in the F1 hybrid, there was limited chromosome pairing: 0-2 bivalents and 20-24 univalents were recorded at diakinesis and 0-1 bivalents at metaphase 1(Table 1). In majority of the cells (95.33%), chromosomes of *O. sativa* and *O. brachyantha* remained unpaired showing lack of homoeology between *O. sativa* and *O. brachyantha* (Figure 2).

The *O. sativa* chromosomes were larger in size and uniformly stained. In anaphase-1, all the cells examined showed unequal distribution of chromosomes. Restitution nuclei were observed in 43% of the pollen mother cells (PMCs). Timing imbalance was noticed in the hybrid, that is, while 12 chromosomes of one species were at metaphase, the chromosomes of other species were moving to the pole (Figure 3).

This indicates that the meiotic cycle of one genome is much shorter than the other genome or the presence of one genome may hasten the condensation of other genome. Limited pairing of chromosomes indicated that the two genomes are highly divergent. Multipolar cells (54.5%) were observed at telophase. Leggard chromosomes were observed in 33% of the cells demonstrating lack of homoeology between *O. sativa* and *O. brachyantha*.

Pollen sterility is one of the indications of the level of relationship of the genomes in the hybrid. Pollen sterility was examined in F1 hybrid and compared to their respective parents. Data on pollen and spikelet sterility is given in Table 2. The parents had 10.0 to 10.4% pollen sterility: the hybrid was highly sterile with 100% pollen sterility. The pollen and spikelet sterility may be due to irregular meiosis or unfavorable gene interaction and genomic disharmony. For assessing the genomic relationship, single strategy may not provide sufficient data to draw conclusion for genomic affinity. A diploid hybrid showing high pairing with pollen and seed sterility indicates that the parental genomes involved may be closely related and the sterility of the hybrid may be due to genetics and not chromosomal factors. Cultivated rice *O. sativa* (2n = 24) and *O. glaberrima* (2n = 24) hybridize

Table 1. Meiotic behavior of wide cross derivatives of rice.

Parameters		*O. sativa*		*O. brachyantha*		*O. sativa* x *O. brachyantha*	
		DK	**M1**	**DK**	**M1**	**DK**	**M1**
Cell analyzed (no)		75	72	151	64	98	150
Univalent							
Range		0 - 2	0 - 2	0 - 4	0 - 4	16 - 24	16 - 24
Mean/cell		0.11	0.21	0.04	2.09	22.12	22.48
Bivalent							
Range		11 - 12	9 - 12	10 - 12	10 - 12	0 - 4	0 - 4
Mean/cell		11.95	11.98	11.89	10.94	0.79	0.73
	0II	-	-	-	-	55.00	56.00
	1II	-	-	-	-	19.38	19.33
	2II	-	-	-	-	19.38	19.33
	3II	-	-	-	-	04.08	01.33
	4II	-	-	-	-	02.04	02.00

DK = Diakinesis; M1 = metaphase 1.

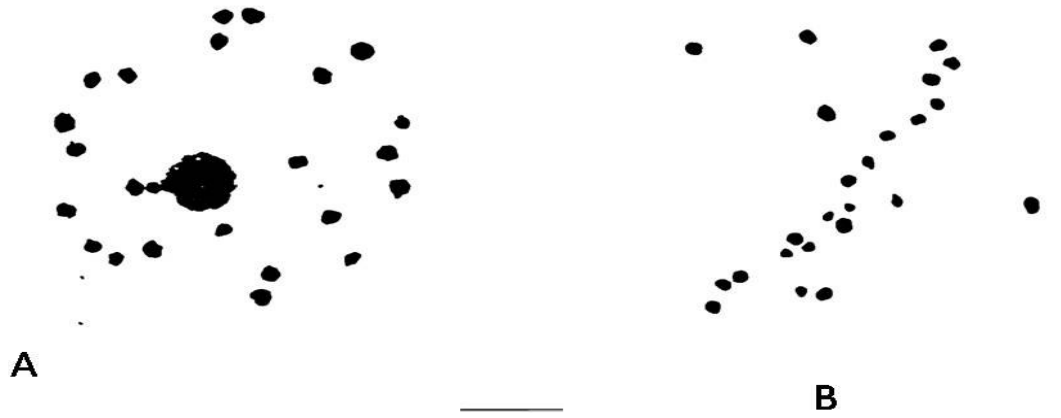

Figure 2. Meiotic chromosome behavior of F1 hybrid (*O. sativa* × *O. brachyantha*). A) Diakinesis showing 24 univalents; B) metaphase-anaphase1 showing 20 univalents at equatorial plate and 4 univalents moved to poles.

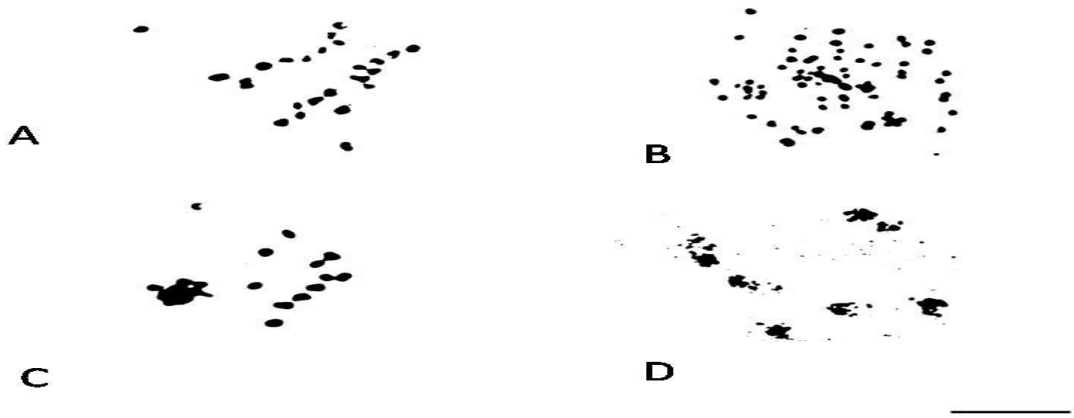

Figure 3. Meiotic chromosome behavior of F1 hybrid (*O. sativa* × *O. brachyantha*). A) Anaphase with unequal distribution of chromosomes; B) restitution nucleus; C) metaphase-telophase indicating the differential condensation of both the genomes; and D) multi-polar cell.

Table 2. Pollen and spikelet sterility in *O. sativa*, *O. brachyantha* and their F1 hybrid.

Parents/Hybrid	Pollen grain			Florets		
	Stained	Unstained	Pollen sterility (%)	Filled	Unfilled	Spikelet sterility (%)
O. sativa	760	85	10.00	1015	135	11.7
O. brachyantha	3840	448	10.4	400	163	28.9
O. sativa x O. brachyantha	1	2111	99.9	0	700	100

very easily and produce vigorous hybrids with almost complete chromosome pairing. However the hybrids are sterile (Bouhamont et al., 1985). Nevertheless the genomes in these species are closely related. The sterility in this hybrid could be due to genetic factors. The F1 hybrid pairing between *Tritium monococcum* and *Tritium urartu* also show complete chromosome pairing (6.97 II/cell), yet the hybrids are completely sterile (Dhaliwal and Jonsson, 1982).

The chromosome pairing in diploid hybrids may suggest a degree of relationship between the parental genomes. However, because diploid hybrids have only two sets of chromosome, they do not offer conditions for preferential pairing. The chromosomes of one genome have the option of pairing only with those of the other genomes. In such circumstances chromosome pairing may not be the function of homology. An erroneous conclusion could be drawn if genomic relationship was based only on chromosome pairing, or sterility/fertility of F1 hybrid without supplementing the data on their DNA sequence homology. Present investigation on genomic affinity between *O. sativa* and *O. brachyantha* were based on three strategies. The data revealed that both genomes belong to different primary gene pool. Abnormal meiosis and lack of chromosome pairing at metaphase-1 could be the main reason of sterility in this hybrid. Thus an understanding of genomic affinities in these species helps to plan effective breeding programs designed to transfer genes from alien species into otherwise superior cultivars of rice.

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