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Full Length Research Paper

Cytological characterization of anther culture derived plants from the interspecific crosses between *Oryza sativa* x *Oryza australinesis* and *Oryza sativa* x *Oryza brachyantha*

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Anther culture is being used in cereal crop improvement both as a source of haploids and for inducing the new genetic variability. We studied the possibilities of producing the aneuploids in rice by anther culture. Anthers for callus induction were plated on semi solid media. Callus formation was observed 20 days after plating. The anthers became necrotic prior to callus formation. Tapei 309 exhibited the highest callus induction efficiency (7.8%) followed by IR31917-45-3-2 (7.7%). IR56 produced (1.45%) callus. The F1 hybrids (IR31917-45-3-2 x *Oryza australiensis*) and IR56 x *Oryza brachyantha* responded poorly for callus induction (0.033%) and (0.030%) respectively. The 879 calli of IR31917-45-3-2 regenerated into six (0.68%) green plants and 79 (8.9%) albinos. All the green plants were haploids. No green plant was produced from the 112 calli of IR56. Similarly, the calli from IR56 x *O. brachyantha* did not show any plant regeneration. The calli from the F1 hybrid (IR31917-45-3-2 x *O. australiensis)* yielded six (20.6%) green and two (6.89%) albino plants. Three of the six plants did not grow after regeneration, while the remaining three plants were used for cytological studies. One plant was aneuploid with 27 chromosomes. Genomic *in situ* hybridization (GISH) using biotin labeled genomic DNA unequivocally detected 14 chromosomes of *O. australiensis* and 13 chromosomes from IR31917-45-3-2 in this aneuploid. Similarly, in the other two plants with 24 chromosomes each, the 12 chromosomes of *O. australiensis* could be discriminated from the 12 chromosomes from IR56.

Key words: Aneuploid, anther culture, *Oryza sativa*, genomic in situ hybridization (GISH).

INTRODUCTION

Cell and tissue culture techniques such as anther culture have attracted considerable attention as supplementary tools to cereal crop improvement (Vasil and Vasil, 1994). It involved the induction of embryoid formation from immature pollen and subsequent regeneration of embryoid into plantlets. Anther culture derived haploids have been used to produce homozygous lines. The application of modern method of gene transfer into crop plants also depend on the development of efficient system for regeneration of full plants from cultured cells and tissue (Vasil and Vasil, 1994). The development of efficient system for regenerating haploid callus culture may enable direct gene transfer into rice. Chromosome doubling after the incorporation of a transgene should facilitate its stable incorporation. Improvement of agronomic traits such as

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Abbreviations: FISH, Fluorescence *in situ* hybridization; **BSA,** bovine serum albumin; **FITC,** fluorescein isothiocynate; **DAPI,** 4',6-diamidino-2-phenylindole; **GISH,** genomic *in situ* hybridization.

development of earliness, increased grain weight, superior grain quality, disease resistance (Zang et al., 1984; Zang, 1989), dwarf plant type and abiotic stresses tolerance have been achieved successfully using anther culture (Alejar et al., 1995).

Anther culture in rice has been improved substantially. However, detail study on wild relatives and their F1 hybrids was extremely limited. Low anther culture response, high percent of albino plantlet regenerations are the principal constraints in establishing anther culture in rice (Roy and Mandal, 2005). We reported the production of haploids, double haploids and aneuploid in rice by using anther culture of wide-crosses. Fluorescence *in situ* hybrid-dization (FISH) a powerful technique for charac-terizing anther culture derived progenies of wide-hybrids, chromosome pairing (Abbasi et al., 2009), assessment of genomic relationship (Abbasi et al., 2010) was used to identify the parental genomes in anther culture derived plants from wide-hybrids.

MATERIALS AND METHODS

Panicle collection and pre-treatment

Panicles were collected at booting stage having a distance of 4 to 8 cm between the flag leaf and the leaf below it. These were washed thoroughly with tap water, wrapped in paper towels, moistened with distilled water and subjected to cold treatment in an incubator at 8°C for 8 days. Some of the panicles were used without treatment.

Callus induction

The panicles were surface sterilized in 20% chlorox for 20 min. Anthers with pollen grains at mid uni-nucleate to early bi-nucleate stages were plated in the callus induction media. Florets were removed from the panicles and cut at their base. Anthers were transferred to semi-solid medium by tapping cut florets. Three media: MS (Murashige and Skoog, 1962), N6 (Chu et al., 1975) and FJ4, a modified MS medium were used for callus induction.

Plant regeneration

All procedures were conducted under aseptic condition using a laminar flow cabinet. Calli were transferred to four modified MS media: M5, M6, SK11 and SK11M and incubated under 16 and 8 h day/night for approximately 30 days. Green plantlets having well developed shoots were transferred to half MS medium without hormones. Roots of the regenerated plantlets were washed with distilled water to remove the calli and medium residues. The rege-nerated green plantlets were transferred to the phytotron (29 and 21°C) in soil in small pots. Callus induction and plant regeneration efficiency were calculated as reported by Abbasi (1999).

$$\text{Callus induction (\%)} = \frac{\text{No. of anthers forming callus}}{\text{Total no. of anthers plated}} \times 100$$

$$\text{Plant regeneration (\%)} = \frac{\text{No. of calli producing green plants}}{\text{Total no. of calli transferred on regeneration medium}} \times 100$$

Mitotic chromosome preparation

Newly emerged roots (1 to 2 cm) from field grown F1 hybrid plants were excised and treated with 5 mM 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 tempe-rature. 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 tri-sodium citrate di-hydrate, pH 4.6). Meristematic portion of root tips were subjected to enzymatic maceration, 3% cellulase (Onozuka R10) + 2% pecto-lyase (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.

Preparation of genomic DNA

The genomic DNA was isolated from 5 to 10 g fresh leaves from *Oryza australiensis* and *Oryza 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 (GISH)

The hybridization mixture containing 120 ng of biotinylated probe, 50% formamide, 3 µg SSS DNA, 2xSSC and 2.4 µg unlabeled *O. sativa* DNA was denatured at 80°C for 10 min and immediately quenched in ice for 5 min. An aliquot of 18 µl was dropped on each slide and 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. For FISH analysis an aliquot of 100 µl of blocking solution, containing 5% bovine serum albumin (BSA) in 4xSSct (4xSSC + 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, 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 2xSSC for 10 min each at 37°C, dehydrated in ethanol series: 70, 95 and 100% for three minutes each at room temperature. The chromosomes were counterstained with 4',6-diamidino-2-phenylindole (DAPI), 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 filter set no. 05, 09 and 25. Photographs were taken with Kodak Ektacolor, ASA/ISO 400.

RESULTS AND DISCUSSION

Callus induction

Anthers for callus induction were plated on semi solid media. Callus formation was observed 20 days after

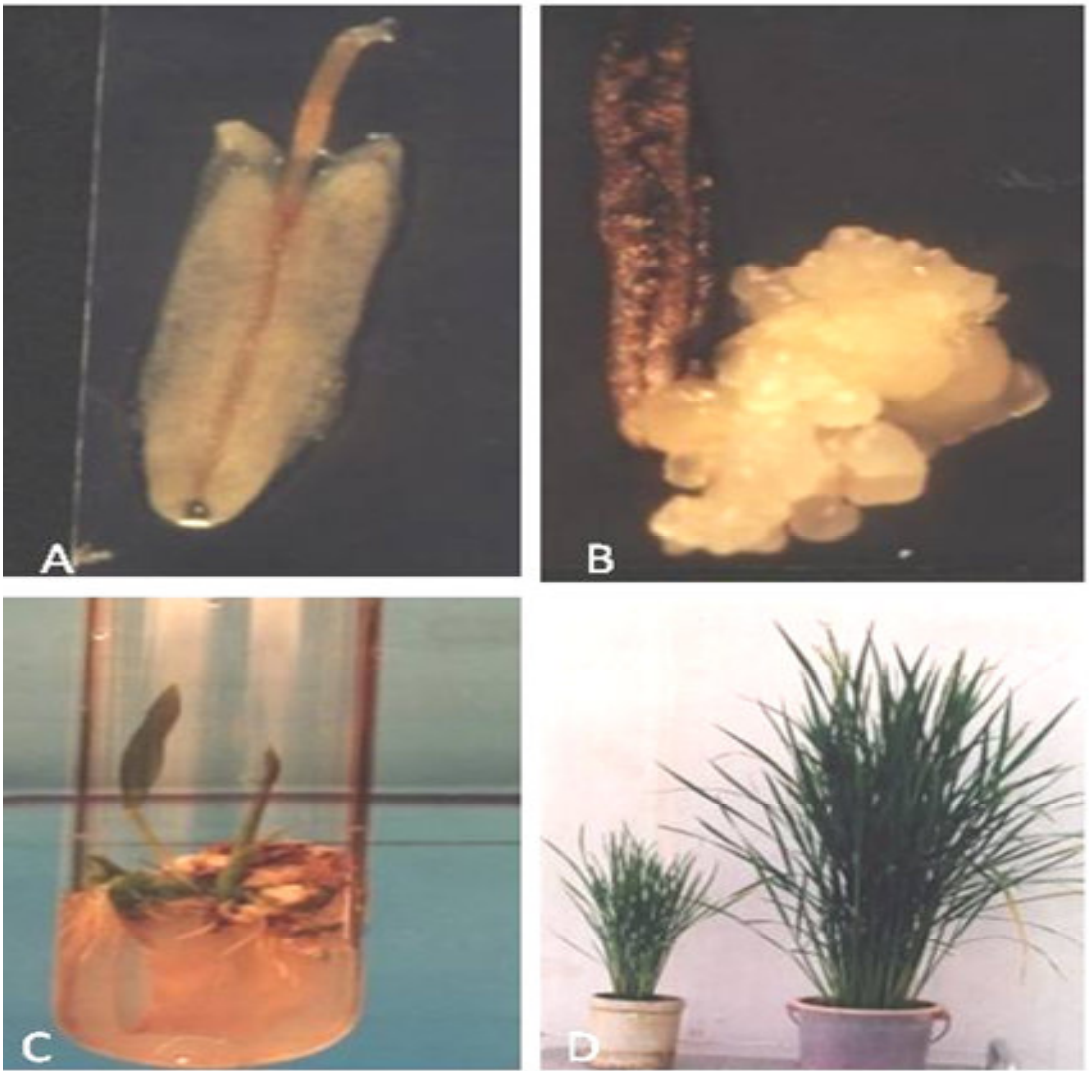

Figure 1. Anther culture of interspecific crosses between *O. sativa* and *O. ausraraliensis*. (A) Anther turned brown before callus induction; (B) same anther as in A producing callus; (C) green plant regenerated; (D) haploid plant (dwarf) and dihaploid (tall).

plating. The anthers become necrotic prior to callus formation (Figure 1 a and b). Tapei 309 exhibited the highest callus induction efficiency (7.083%), followed by IR31917-45-3-2 which gave the highest response (4.597%). IR56 produced (1.463%) callus. The F1 hybrids (Ir31917-45-3-4 x *O. australiensis*, IR56 x *Oryza brachyanatha*) responded poorly to callus induction (0.033%) and (0.03%) (Table 1).

Among the green plants, five plants (14.3%) were fertile. Similarly, 879 calli of IR31917-45-3-2 regenerated into six (0.68%) green and 79 (8.9%) albinos. All the green plants were haploids. No green plants were produced from the 112 calli of IR56. The calli from the F1 hybrid, IR31917-45-3-2 x *O. australiensis* yielded six (20.6%) green (Figure 1d) and two (6.89%) albino plants. Three of the six plants did not grow after regeneration, while the remaining three plants were used for cytological studies. The calli from IR56 x *O. brachyantha* did not show any plant regeneration (Table 2).

Cytological characterization of anther culture derived plants

Three anther culture derived plants from the F1 hybrid *O. sativa x O. australiensis* were used for GISH analysis. One plant had 27 chromosomes whereas, other two plants showed 24 chromosomes. Genomic DNA from *O. australiensis* was used as a probe. The biotin labeled probe produced uniform labeling pattern over the entire length of all the 14 *O. australiensis* chromosomes (dark brown) whereas, 13 chromosomes of *O. sativa* appeared

Table 1. Callus induction from anther culture of wide-cross derivatives of rice.

Genotype	Anthers plated (no)	Callus produced (no)	Callus induction (%)
Tapei 309	10914	773b	7.083a
IR31917-45-3-2	28260	1299a	4.597b
O. australiensis	3720	0 e	0 e
IR56	7650	112c	1.463c
IR31917-45-3-2 x *O. australiensis*	88837	29d	0.033d
IR56 *x O. brachyantha*	53670	15de	0.030d

Mean followed by same letter do not differ significantly.

Table 2. Plant regeneration from anther derived calli of wide-cross derivatives.

Genotype	Callus transferred (no)	Plant regeneration			
		Green	(%)	Albino	(%)
Tapei 309	423	35a	8.30 b	133a	31.4 a
IR31917-45-3-2	879	06 b	0.68 c	79b	08.98b
IR56	112	00 c	0.00 c	04 c	03.57cd
IR31917-45-3-2 x *O .australiensis*	29	06 b	20.60 a	02cd	06.89bc
IR56 x *O. brachyantha*	15	00 c	0.00c	00d	00.00d

Mean followed by same letter do not differ significantly from each other.

light blue after Giemsa staining (Figure 2a).

For FISH analysis when somatic metaphase cells were stained with DAPI, all the 27 chromosomes fluoresced blue under UV light excitation. In the same cell, the *O. australiensis* chromosomes showing the hybridization signal, appeared green under blue light excitation allowing the identification of all the *O. australiensis* chromosomes whereas, *O. sativa* chromosomes appeared blue due to counterstaining with DAPI (Figure 2b).

FISH analysis of the two other plants (2n = 24) was carried out. In a somatic metaphase cell stained with DAPI, all the 24 chromosomes fluoresced blue. After *in situ* hybridization 12 *O. australiensis* chromosomes fluoresced green under blue light excitation due to FITC and unlabeled *O. sativa* chromosomes appeared blue due to counterstaining with DAPI (data not shown). This indicated that, these labeled chromosomes were from *O. australiensis*. Similarly, both the parental chromosomes were clearly discriminated showing labeled *O. australiensis* chromosomes (green) and unlabeled *O. sativa* chromosomes. No restructured chromosome could be identified among 100 cells analyzed through *in situ* hybridization.

Anther culture is being used in rice breeding program both as a source of haploids and new genetic variation. We studied the anther culture ability and chromosomal variation of wide hybrids and their parents using different media formulation. Significant genotypic differences were observed for callus induction. The genotype of the donor plant affects anther culture response (Moieni and Sarrafi, 1995). Nuclear and cytoplasmic control of anther culture has been reported (Ekiz and Konzak, 1991). Similar observations were recorded in this study. No green plants were produced both in IR56 and the hybrid between IR56 and *O. brachyantha*. IR3191745-3-2 has high callus induction and green plant regeneration ability compared with IR56. Similar trends of callus induction and green plant regeneration was observed in their respective hybrids. The low yield of haploid green plants from rice anther culture and albinos are serious problems. Even after culturing 3720 anthers, we did not obtain even a single callus in *O. australiensis* and major portions of the regenerants in other genotypes were albinos. Albinos' plants do not contain mature chloroplast and large scale deletion of the plasted genome in the microspore-derived plants of rice has been reported (Jahne and Lorz, 1995). The anther culture media is reported to be a major factor for anther culture response for callus induction and regeneration of plantlets (Fadel and Wenzel, 1990). In this study, MS medium gave the better response for callus induction followed by FJ4 and N6.

Aneuploidy as the chromosomal abnormality has been observed frequently in cell culture. The level of ploidy in our regenerants varied from 2n = 24 to 2n= 27. The chromosomal changes involving multiplication of entire genomes or some chromosomes have been reported in callus of wheat (Wang and Hu, 1985). In this study, the fluorescent GISH revealed 13 chromosomes from *O. sativa* and 14 chromosomes from *O. australiensis* in the regenerant with 27 chromosomes. The preponderance of E genome chromosomes may be related to their predominately heterochromatic nature. Heterochromatin is

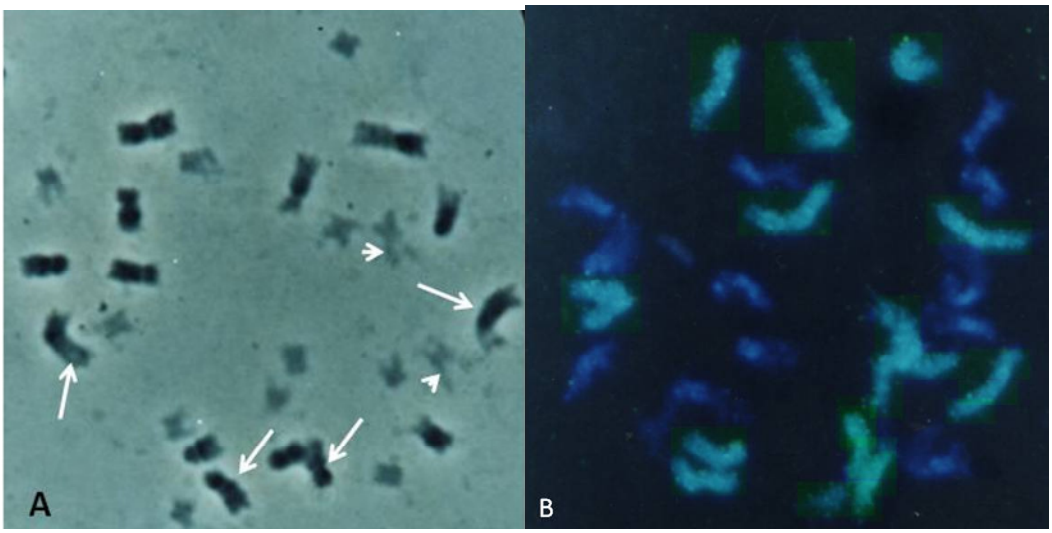

Figure 2. Genomic *in situ* hybridization to root tip chromosome preparation from anther culture derived plant with 27 chromosomes. Larger and darkly stained chromosomes are from *O. australiensis*, smaller and lightly stained chromosomes are from *O. sativa*. (A) Arrow shows the duplicated chromosomes from *O. australiensis* and arrow head shows the duplicated chromosomes from *O. sativa*; (B) FISH showing 13 chromosomes from *O. sativa* (blue) and 14 larger chromosomes from *O. australiensis* (green).

known to replicate later than euchromatin, that is why the E genome undergo non-disjunction mechanism by which they multiply. We do not observe any structural abnormality. We have previously reported GISH as a powerful technique for characterizing parental genomes in the wide hybrids (Abbasi et al., 2010), localizing introgression on the chromosomes (Abbasi et al., 2010), detecting genomic affinity (Abbasi et al., 2010) and assessing genomic relationship (Abbasi et al., 2010). Results showed that aneuploidy could be developed by anther culture in rice.

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