HOSTED BY

ELSEVIER

Available online at www.sciencedirect.com

ScienceDirect

Rice Science, 2015, 22(6): 290–299

Development and Characterization of Elite Doubled Haploid Lines from Two Indica Rice Hybrids

Rukmini MISHRA[1], Gundimeda Jwala Narashima RAO[1], Ravi Nageswara RAO[1], Pankaj KAUSHAL[2]

([1]*Crop Improvement Division, Central Rice Research Institute, Cuttack 753006, India;* [2]*Indian Grassland and Fodder Institute, Jhansi 284003, India*)

Abstract: Despite significant yield advantage over inbred rice, the adoption rate of hybrid rice in India is very low due to the high seed cost and poor quality of the produce. To alleviate the problem, we initiated a doubled haploid (DH) breeding approach to develop new lines from two elite indica rice hybrids (CRHR5 and CRHR7) through rapid fixation of homozygosity in the recombinants. *In vitro* culture of the rice anthers resulted in 243 and 186 fertile DH lines of CRHR5 and CRHR7, respectively. Flow cytometric and pollen fertility analyses confirmed the DH ploidy status of the regenerations. Morpho-agronomic evaluation revealed 100% uniformity and stability for all the characters in the DH lines of both hybrids. Nineteen promising DH lines of each hybrid were advanced to A_2 generation for yield evaluation. The yield levels of the DH lines ranged from 5 097–6 965 kg/hm^2 for CRHR5 and 5 141–7 235 kg/hm^2 for CRHR7, which were at par or higher than the parental hybrids. Physico-chemical characterization and cooking quality analyses revealed significant and acceptable values for grain length and width, alkali spreading value, amylose content and water uptake ratio of the selected DH lines. Three DH lines, CR5-10, CR5-49, CR5-61 from CRHR5, and four DH lines, CR7-5, CR7-7, CR7-12 and CR7-52 from CRHR7, showed significant grain yield and quality characteristics and have been recommended for multi-location trials for subsequent release as new indica doubled haploid rice varieties.

Key words: anther culture; doubled haploid; indica rice hybrid

Rice (*Oryza sativa* L.), one of the most important crops in the world, is providing a carbohydrate source for over half of the world's population (Feng et al, 2013). As the global population is expected to reach around 8.5 billion by 2025, it will be necessary to produce 50% more food by then. With gradual reduction in cropland area, it is imperative to increase productivity levels for unit area. Hybrid rice, which can offer significant yield advantages over inbreds, has revolutionized the rice productivity and production in the world. More than 50% cultivated rice area is under hybrid rice cultivation in China. However, the adoption rate of hybrid rice technology in India is poor due to some problems such as high seed cost and poor grain quality of the produce (Mishra et al, 2013). Anther culture, an innovative method for hastening the generation of homozygous doubled haploid (DH), can be used to accelerate the varietal improvement programs in rice (Herawati et al, 2010). The recombinant doubled haploids derived from hybrid rice with yield at par the parent hybrids and better grain quality, can circumvent the problems associated with hybrid rice technology.

Enormous advances have been made throughout the world since the valuable discovery of androgenic haploidy by Guha and Maheshwari (1964) and production of rice haploids by Niizeki and Oono (1968). More than 20 rice varieties have been reported through DH approach in China, Korea, Japan, and USA (Zepata-Arias, 2003). Though anther culture has been recognized as a valuable tool in plant breeding programs, its application is limited due to difficulties in the induction of embryogenic calli from some genotypes (Lee and Lee, 2002). In general, indica rice has low anther

Received: 22 May 2015; **Accepted:** 6 July 2015

Corresponding author: Rukmini MISHRA (rukmini.mishra@gmail.com)

Peer review under responsibility of China National Rice Research Institute

http://dx.doi.org/10.1016/j.rsci.2015.07.002

culturability (Dewi et al, 2009) and the recalcitrance of them relates to early anther necrosis, poor callusing ability, low plant regeneration, and frequent regeneration of albino plant (Grewal et al, 2009). Several attempts at anther culture of indica rice have also met with limited success (Ratheika and Silva, 2007; Silva and Achala, 2008). In indica rice, success using the doubled haploid approach was demonstrated recently through the development of two varieties, Satya Krishna and Phalguni, at Central Rice Research Institute.

The present study aims to generate elite doubled haploids from two elite indica hybrids rice CRHR5 and CRHR7. Further, the systematic evaluation and isolation of the doubled haploids with high yield and good grain quality was discussed.

MATERIALS AND METHODS

Rice materials

Four elite hybrid rice namely CRHR5 (CRMS32A/IR42266-29-3R), CRHR7 (CRMS31A/IR42266-29BR), JKRH401 (RV-2A/RV-44R) and JKRH405 suitable to different rice ecologies were taken for the study. JKRH401 and JKRH405 were developed by JK Agric Genetics Ltd, Hyderabad, India, while CRHR5 and CRHR7 were developed at Central Rice Research Institute, Cuttack, India. Twenty-five days old seedlings, raised in dry seed beds under ideal conditions, were transplanted in a well puddled field with 20 cm × 15 cm (between and within rows) spacing. Recommended doses of N:P:K (90:60:60) were applied in three split doses and based crop protection measures were undertaken. At the booting stage, the panicles from the primary and secondary tillers were collected and subjected to cold treatment at (8 ± 2) °C for 8–10 d in a refrigerator.

Anther inoculation and callus induction

Pretreated spikelets were surface sterilized using 4% sodium hypocholide (NaClO) for 5 min and rinsed three to four times with sterile deionized water. Selection of the spikelets was based on cytological observation, size and the position of the anther. Anthers were isolated by cutting the basal end of the florets and dusted uniformly over the surface of the medium by grasping cut floret as open end down and tapping it on the rim of the culture tube so that all the anthers were dropped onto the surface of the culture medium. The inoculated anthers were incubated in dark at (25 ± 1) °C, and the observation on the anther response to callus induction was recorded 3–4 weeks after inoculation.

Media

Three media having different nutritional compositions namely N6 (Chu et al, 1975), MO19 (Raina and Zapata, 1997), and SK-1 (Raina, 1989) were employed. In addition, all the three media (pH = 5.8) were supplemented uniformly with sugar (maltose 30 mg/L), two phytohormones [2 mg/L of 2,4-dichlorohpenoxyacetic acid (2,4-D) and 0.5 mg/L kinetin] and 100 mg myo-inositol, and agar (0.8%) were used for solidification. The media was dispensed at the rate of 20–25 mL per culture tube (25 mm × 150 mm) and sterilized through autoclaving at 15 pounds per square rich for 18 min.

Plant regeneration and transformation

Embryogenic calli of 1–3 mm size were transferred to Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 0.25 mg kinetin, 0.75 mg benzylaminopurine (BAP) and 0.25 mg 1-naphthaleneacetic acid (NAA) for shoot regeneration. Cultures were incubated with a 16-h light/8-h dark regime at (25 ± 10) °C under artificial light (2000 lux). Green shoots with 2–3 cm length were transferred to MS medium supplemented with kinetin (0.25 mg) and NAA (1.00 mg) for vigorous root development. The regenerations with well-developed roots were transferred to green house and grown till maturity.

Ploidy analysis of anther derived plant

The regenerations were grouped into haploids, diploids and tetraploids based on their morphology and floral characters, cytological analysis, flow cytometry, and pollen fertility. For cytological study, young spikelets at the suitable stage were fixed in acetic acid-alcohol (1:3) mixture for 24 h, and pollen mother cells at the meiotic division stage were stained using smearing technique with 1% aceto-carmine stain solution. For pollen fertility analysis, un-pollinated spikelets before flowering from different samples were fixed in formaldehyde, acetic acid and 70% alcohol solution (1:1:18), then crushed on a slide and stained with potassium iodide or 2% aceto carmine solution, and observed under bright field microscope for estimation of pollen fertility. For flow cytometry (BD FACSCanto 11, BD Biosciences, San Jose, CA), young leaves were chopped with sharp razor blade for isolation of nuclei in 2 mL extraction buffer (pH = 7.0) containing 100

mmol/L Tris-HCl, 5 mmol/L $MgCl_2$, 85 mmol/L NaCl, 0.1% Triton-X 100 and 1 μg/mL of 4,6-diamidino-2-phenylindole. The suspension was then filtered through nylon filters with 30 μm wild mesh and incubated for 10–15 min before the measurement in flow cytometer. Results were recorded with the software CA32.14/2004. Each sample was analyzed for three times and the ploidy status was deduced from resultant histogram based on three replications.

Morpho-agronomic evaluation of doubled haploids

A total of 243 DH lines of CRHR5 and 186 lines of CRHR7 were evaluated for different morpho-agronomic traits in A_1 and A_2 generations under field conditions. Three and eight rows constituted a replication in A_1 and A_2 generations and the respective parental hybrids (CRHR5 and CRHR7) and Lalat and Tapaswini, two high yield inbred varieties, were used as controls in each generation. Based on their performance in A_1 generation, promising entries of each hybrid were advanced to A_2 generation for further evaluation. Data were collected from five random plants for morphological traits, and two samples of 1 m^2 area from each replication for yield while the 1000-grain weight was recorded from three samples.

Physico-chemical characteristics and cooking quality analysis

The physico-chemical characteristics such as hulling, milling, head rice recovery, grain length and width, alkali spreading value, amylose content, gel consistency and cooking characteristics including volume expansion, elongation ratio, and water uptake were evaluated for eight selected DH lines with high yield from both the varieties according to the protocol of Bhonsle and Sellappan (2010).

Statistical analysis

All the statistical analyses were carried out using IRRISTAT (IRRI, 2005) and INDOSTAT (INDOSTAT, 2004) programs. Analysis of variance (ANOVA) for each character was carried out based on the means. It was performed to compare the yield and yield components of the entries by using the least significance difference (LSD) test. The total variances were partitioned into variance due to replication, treatment and error. *F*-test was used to test the significance of difference among the treatments. LSD test was used to show the variation between mean values of different parameters. Standard error of means and coefficient of variation were also calculated.

RESULTS

Optimization of nutritional requirements for hybrids

The optimization of nutritional requirements was done to study the anther culture response of different heterotic indica hybrids. ANOVA revealed significant differences ($P = 0.001$) among the three media employed in all the hybrids for all the parameters, i.e. callus induction, callus regeneration, green plant regeneration and albino plant regeneration frequencies (Table 1). Out of the three media employed, N6 medium was found to be the best media for all the hybrids followed by MO19. On individual basis, CRHR5 and CRHR7 had responded well on N6, JKRH405 responded well on MO19 while JKRH401 responded well on SK-1

Table 1. Level of anther culture response for different parameters observed on three different media. %

Parameter	Hybrid	Medium		
		N6	MO19	SK-1
Callus induction	CRHR5	34.56 ± 0.33	31.62 ± 0.32	25.58 ± 0.18
	CRHR7	30.14 ± 0.35	26.54 ± 0.23	27.44 ± 0.22
	JKRH401	16.37 ± 0.25	20.16 ± 0.35	23.53 ± 0.37
	JKRH405	28.34 ± 0.29	24.45 ± 0.22	22.86 ± 0.28
Callus regeneration	CRHR5	37.23 ± 0.26	33.43 ± 0.21	27.31 ± 0.22
	CRHR7	35.43 ± 0.37	29.23 ± 0.22	29.11 ± 0.38
	JKRH401	22.66 ± 0.33	19.29 ± 0.42	25.77 ± 0.43
	JKRH405	28.44 ± 0.20	29.33 ± 0.24	27.92 ± 0.41
Green plant regeneration	CRHR5	21.12 ± 0.20	17.81 ± 0.17	15.36 ± 0.29
	CRHR7	19.56 ± 0.33	15.42 ± 0.29	13.23 ± 0.27
	JKRH401	10.28 ± 0.46	8.35 ± 0.51	12.18 ± 0.31
	JKRH405	11.43 ± 0.37	14.02 ± 0.51	13.02 ± 0.37
Albino plant regeneration	CRHR5	17.11 ± 0.18	15.73 ± 0.33	12.03 ± 0.21
	CRHR7	15.87 ± 0.14	13.82 ± 0.40	15.78 ± 0.37
	JKRH401	12.38 ± 0.31	10.94 ± 0.27	13.59 ± 0.20
	JKRH405	17.01± 0.41	15.31 ± 0.44	15.00 ± 0.39

medium. MO19 and SK-1 had moderate level of callus regeneration, while the green plant regeneration was comparatively low in both the two media.

Characterization of anther culture derived plants

Ploidy analysis based on morphological characteristics of the regenerations revealed that the haploid plants were fully sterile, with short stature and small glumes. Doubled haploids looked like normal diploid plants and were fully fertile. Tetraploids have larger spikelets with awns and less number of grains/panicles and generally semi-sterile (Fig. 1-A and -B). The ploidy status of regenerated plants was confirmed by flow cytometric analysis. Each sample was analyzed in three replications and ploidy estimation was deduced from resultant histogram. A typical DNA histogram with prominent peaks representing the genome size of the samples was observed, and different ploidy levels were clearly represented by different peaks (45, 90, 135 and 180 at *x* axis) representing the genome size (1×, 2×, 3× and 4×) of the samples (Fig. 1-C).

Cytological characterization of pollen mother cells revealed four different ploidy levels, i.e. haploid, diploid, triploid and tetraploids. The meiotic behaviour of haploid, diploid, triploid and tetraploids derived from the regenerations is represented in Fig. 2-A to -D. Pollen fertility was carried out to observe the segregation pattern of pollen size of anther derived plants. The pollen grains of haploids were comparatively small in size and did not take the stain showing zero fertility (Fig. 2-E). The fertility percentage of double haploids was observed to be always higher than that of the tetraploids. In case of DHs, the size of the pollen was medium and fully filled pollen grains were viewed under the microscope (Fig. 2-F). The pollen size of triploids was comparatively larger than that of the doubled haploids and the pollen grains were partially stained (Fig. 1-G). Tetraploids had a range of 5%–50% pollen fertility and partly stained pollen grains were observed under the microscope (Fig. 2-H). The result indicated that the pollen grain size was positively correlated with ploidy level.

Ploidy analysis of regenerations

Anther culture resulted in a total of 1 159 plants from the four hybrids. Ploidy analysis revealed 243 (49.0%) regenerations as DHs and 221 (44.6%) regenerations as haploids from CRHR5 while 186 (41.2%) regenerations as DHs and 167 (45.9%) regenerations as haploids from CRHR7 (Supplemental Table 1). JKRH405 and JKRH401 showed poor regeneration

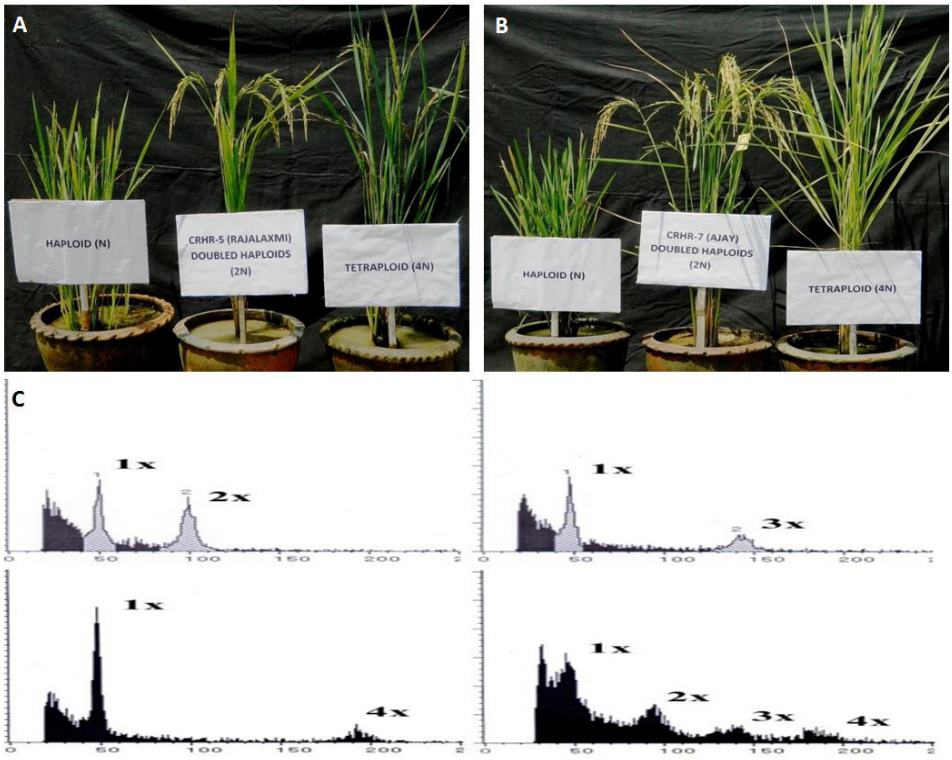

Fig. 1. Morphological characterization of anther derived regenerates (A, B) and flow cytometric analysis of anther derived regenerates (C).

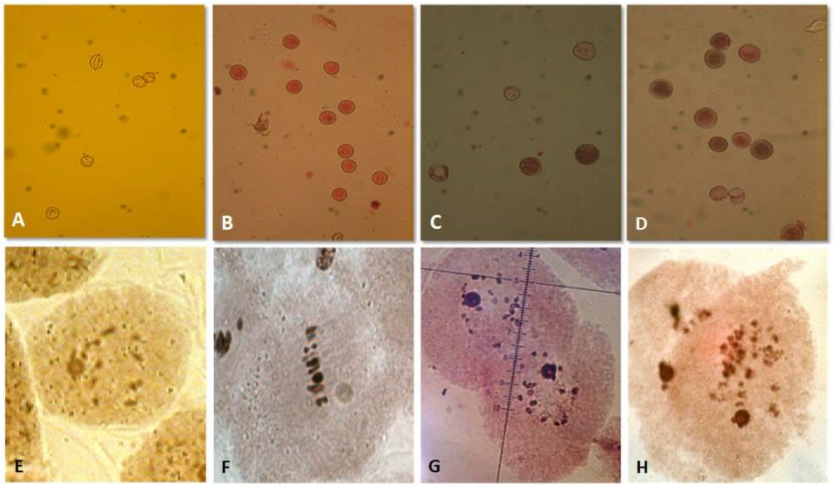

Fig. 2. Pollen fertility analysis of anther derived regenerate (A, Haploid; B, Diploid; C, Triploid; D, Tetraploid) and cytological characterization of anther derived regenerate (E, Haploid; F, Diploid; G, Triploid; H, Tetraploid).

with 162 and 97 green plants, respectively. While CRHR5 and CRHR7 regenerated more doubled haploids, JKRH405 and JKRH401 resulted in more number of haploids. The proportion of tetraploids in the four genotypes is in the range of 6.3% (CRHR5) to 16.6% (JKRH405). Therefore, the DHs from CRHR5 and CRHR7 were used for further analysis while those from JKRH401 and JKRH405 were rejected.

Agronomic performance of doubled haploids

A wide range of segregation was seen in the progeny of the elite hybrids studied in A_1 generation. The range of segregation of six important agronomic traits, i.e. duration, plant height, panicle length, tiller number, 1000-grain weight and yield are represented in Fig. 3. The frequency distributions of the six agronomic traits for DHs from CRHR5 and CRHR7 revealed uniform distribution of recombinants with some values on the higher side of the parent and some on the lower side. (Supplemental Figs. 1 and 2). Most of the doubled haploids (97%) were observed to be completely homozygous for all the agronomical characters studied. Ninety-two percentage DH lines from CRHR5 had shorter plant height than CRHR5. For 1000-grain weight, the distribution was skewed towards lower side and many of the DH lines had less 1000-grain weight values than CRHR5. Likewise, for DHs from CRHR7, 70% recombinants had higher crop duration as compared to CRHR7. Similarly, 80% of the DHs had shorter plant height and 86% had lower 1000-grain weight than CRHR7.

A selected set of 19 DHs from CRHR5 and CRHR7, respectively were subjected to morpho-agronomic evaluation in A_1 and A_2 generation (Table 2). Overall, the analysis of variance for agronomic performance of DH lines from CRHR5 and CRHR7 revealed no significant differences among the replications

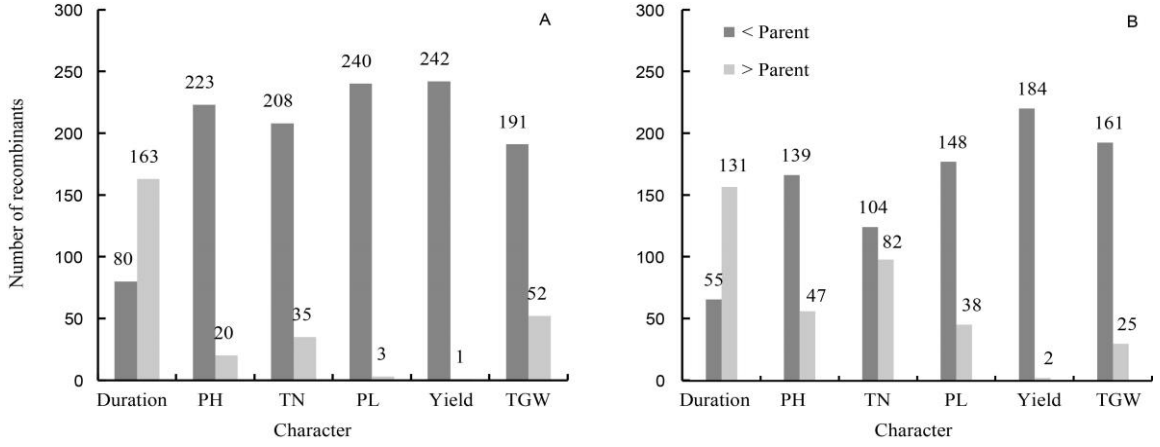

Fig. 3. Characters in recombinant doubled haploids of CRHR5 (A) and CRHR7 (B) in A_1 generation.

PH, Plant height; TN, Tiller number; PL, Panicle length; TGW, 1000-grain weight.

Table 2. Agronomic performance of selected anther derived doubled haploids from CRHR5 and CRHR7 in A_1 and A_2 generations.

Line	Generation	Duration (d)	PH (cm)	TN	PL (cm)	TGW (g)	Yield (kg/hm^2)	Line	Generation	Duration (d)	PH (cm)	TN	PL (cm)	TGW (g)	Yield (kg/hm^2)
CR5-6	A1	120	97	7	24	23.97	5 973	CR7-2	A1	136	116	12	28	18.12	6 864
	A2	122	98	7	26	22.78	6 233		A2	138	117	9	30	19.16	6 497
CR5-8	A1	145	92	12	23	20.95	5 610	CR7-5	A1	142	126	13	28	20.15	7 210
	A2	147	90	12	23	20.60	5 500		A2	142	119	11	27	20.18	6 937
CR5-9	A1	145	94	11	25	22.75	5 841	CR7-7	A1	141	111	10	32	22.60	7 345
	A2	145	93	10	26	22.25	5 760		A2	140	103	9	33	21.28	7 235
CR5-10	A1	134	103	8	25	22.08	6 864	CR7-12	A1	142	122	11	30	21.20	7 010
	A2	136	98	11	25	21.51	6 953		A2	142	114	8	31	20.87	7 042
CR5-19	A1	128	90	8	25	24.00	7 012	CR7-17	A1	141	102	9	27	25.35	6 567
	A2	130	94	9	26	23.99	6 510		A2	144	101	8	27	24.98	6 103
CR5-21	A1	136	89	12	25	25.80	6 270	CR7-22	A1	142	106	10	30	21.80	6 534
	A2	134	90	12	27	25.26	5 987		A2	140	108	7	30	21.15	6 110
CR5-41	A1	145	90	12	26	26.48	6 006	CR7-25	A1	140	112	12	25	19.14	6 600
	A2	144	92	11	27	26.08	5 757		A2	138	113	8	28	19.74	6 086
CR5-49	A1	140	105	10	27	24.60	7 210	CR7-27	A1	122	106	8	27	22.97	6 336
	A2	142	110	12	30	24.10	6 937		A2	126	102	8	27	21.58	6 317
CR5-50	A1	140	92	7	24	23.27	6 765	CR7-40	A1	136	107	11	31	23.04	6 831
	A2	144	93	7	24	23.14	6 130		A2	137	101	9	31	23.04	6 422
CR5-52	A1	136	84	11	25	21.40	6 039	CR7-41	A1	132	96	7	25	23.79	5 940
	A2	138	82	10	25	21.43	5 663		A2	132	97	9	27	23.12	5 240
CR5-53	A1	136	92	5	21	27.28	5 907	CR7-46	A1	147	104	10	27	25.67	5 511
	A2	136	93	7	21	26.99	5 697		A2	145	92	8	27	24.62	5 432
CR5-61	A1	151	95	7	22	20.94	7 226	CR7-48	A1	134	109	6	27	22.04	6 534
	A2	150	112	7	28	21.42	6 965		A2	134	113	9	27	22.19	6 350
CR5-68	A1	134	98	7	25	24.91	6 765	CR7-52	A1	132	104	9	28	26.62	6 450
	A2	135	100	11	26	24.09	5 961		A2	134	116	8	27	25.64	6 817
CR5-75	A1	132	98	7	26	23.93	6 435	CR7-55	A1	120	84	9	21	21.13	5 874
	A2	133	100	8	27	24.08	5 097		A2	128	100	11	25	21.37	6 727
CR5-78	A1	127	102	5	25	19.98	6 633	CR7-57	A1	131	119	10	28	18.54	5 480
	A2	129	110	9	26	20.41	5 637		A2	122	98	10	24	20.77	6 263
CR5-85	A1	131	88	6	23	21.30	6 650	CR7-58	A1	134	108	9	25	24.21	5 940
	A2	130	97	11	26	21.14	6 215		A2	134	115	11	29	19.45	5 617
CR5-105	A1	152	102	10	27	25.05	6 237	CR7-59	A1	136	110	11	25	23.48	5 620
	A2	150	102	10	27	24.76	6 075		A2	138	105	11	27	24.06	5 141
CR5-124	A1	134	108	10	26	24.89	6 600	CR7-62	A1	122	102	9	26	21.67	5 685
	A2	134	118	9	28	24.25	6 138		A2	125	105	9	28	21.40	6 053
CR5-129	A1	128	106	8	28	19.78	5 960	CR7-68	A1	145	111	12	27	25.98	6 730
	A2	127	106	11	29	20.54	5 450		A2	145	110	10	26	25.09	6 447
CRHR5	A1	130	110	10	30	24.10	7 350	CRHR7	A1	134	107	11	28	23.65	7 590
(Parent)	A2	132	107	8	28	24.93	7 648	(Parent)	A2	136	109	12	29	22.49	7 757
Lalat	A1	129	101	9	20	20.10	5 523	Lalat	A1	129	101	10	20	20.10	5 523
(CK)	A2	128	100	9	21	21.55	5 520	(CK)	A2	129	102	8	21	21.89	5 535
Tapaswini	A1	138	96	9	26	20.56	5 530	Tapaswini	A1	136	96	26	9	21.21	5 340
(CK)	A2	136	28	9	26	21.21	5 345	(CK)	A2	138	107	10	24	21.51	5 420
SE	A1	± 1.2	± 1.0	± 0.9	± 0.7	± 0.8	± 4.1	*SE*	A1	± 0.9	± 7.0	± 0.7	± 0.7	± 0.1	± 5.7
	A2	± 1.4	± 1.3	± 0.8	± 0.6	± 0.4	± 6.1		A2	± 1.0	± 2.5	± 1.0	± 0.7	± 0.5	± 6.6
LSD	A1	3.5	3.0	2.5	1.93	0.23	116.7	*LSD*	A1	2.7	2.0	2.1	1.9	0.4	162.1
(P = 0.05)	A2	4.1	3.8	2.3	1.60	1.23	182.8	(P = 0.05)	A2	2.9	7.0	2.9	2.1	1.4	190.0

PH, Plant height; TN, Tiller number; PL, Panicle length; TGW, 1000-grain weight; *SE*, Standard error; *LSD*, Least significant difference.

(Supplemental Tables 3, 4, 5 and 6). All the DH lines recorded high yield levels in both the A_1 and A_2 generations and were at par with the parental hybrids. However, the analysis revealed significant differences in yield (at 1% significance level) between the entries, indicating that DH lines differed from each other in their yield potential. For CRHR5, the yield levels of the DH lines ranged from 5 097–6 965 kg/hm^2 (median 6 763 kg/hm^2, *t*-test; P = 0.00021), which was slightly lower than the parental hybrid (7 648 kg/hm^2) but significantly higher than the control, Lalat (5 520 kg/hm^2) and Tapaswini (5 345 kg/hm^2) in A_2 generation. Prominent DH lines from CRHR5 with high grain yield included CR5-61 (6 965 kg/hm^2), CR5-10 (6 953 kg/hm^2), CR5-49 (6 937 kg/hm^2), CR5-19 (6 510 kg/hm^2), CR5-6 (6 233 kg/hm^2) and CR5-85 (6 215 kg/hm^2). Similarly, the yield levels of DHs from CRHR7 ranged from 5 141–7 235 kg/hm^2 (median 6 342 kg/hm^2, *t*-test; P = 0.00023) and was significantly higher than the control rice varieties.

Table 3. Grain quality, physico-chemical and cooking characteristics of doubled haploid (DH) lines derived from hybrid CRHR5 and CRHR7.

DH line	BRR (%)	MRR (%)	HRR (%)	GLAC	ER	GL (cm)	GW (cm)	L/B	VER	WUR	ASV	Amy (%)
CR5-6	74.51	67.23	59.12	10.03	1.65	6.09	2.34	2.60	3.50	245	6	22.91
CR5-10	78.53	68.51	59.11	9.13	1.60	5.70	1.94	2.94	3.50	235	5	23.62
CR5-19	74.52	57.65	50.54	9.27	1.46	6.34	2.16	2.94	3.25	225	5	23.42
CR5-49	78.43	68.51	60.54	10.04	1.70	5.90	2.22	2.66	3.50	215	7	23.13
CR5-61	78.11	67.88	61.27	8.67	1.35	6.43	2.06	3.12	3.50	235	7	23.73
CR5-85	75.63	65.11	57.36	9.33	1.44	6.49	1.77	3.67	3.50	190	7	22.83
CR5-105	76.53	67.89	63.53	9.57	1.62	5.91	1.75	3.38	3.50	295	6	22.41
CR5-124	77.33	67.34	65.76	10.13	1.55	6.54	2.16	3.03	3.50	160	5	24.65
CRHR5	78.11	65.23	54.12	9.49	1.38	6.23	2.06	3.02	3.50	245	7	26.37
Lalat	77.00	65.50	52.20	10.20	1.53	6.66	2.11	3.15	3.70	227	5	26.60
SE	± 0.06	± 0.20	± 0.05	± 0.06	± 0.50	± 0.07	± 0.04	± 0.04	± 0.10	± 5	± 0.1	± 0.05
LSD (*P* = 0.05)	0.17	0.57	0.14	0.19	0.12	0.16	0.13	0.13	0.37	13.2	3.20	0.14
CR7-2	79.13	63.21	45.80	8.98	1.62	5.55	1.83	3.03	3.50	202	7	22.31
CR7-5	76.54	66.24	58.22	10.45	1.75	5.97	1.91	3.13	3.50	270	7	23.66
CR7-7	77.51	68.58	53.45	11.01	1.69	6.51	2.03	3.21	3.25	265	6	22.39
CR7-12	77.66	69.52	60.32	11.23	1.77	6.33	1.91	3.31	3.45	198	6	22.46
CR7-40	78.58	67.73	60.27	9.31	1.57	5.93	1.91	3.10	3.35	190	7	23.64
CR7-52	76.39	59.27	53.49	9.17	1.51	6.08	2.24	2.71	3.45	243	7	24.63
CR7-55	73.54	63.61	47.37	8.66	1.45	5.99	1.95	3.07	3.50	145	4	24.37
CR7-68	76.57	65.51	55.32	9.24	1.60	5.77	2.27	2.54	3.45	211	4	24.07
CRHR7 (P)	77.21	64.29	54.64	10.25	1.48	6.88	2.21	3.11	3.50	235	7	26.35
Lalat	77.00	65.50	52.20	10.20	1.53	6.66	2.11	3.15	3.60	227	5	26.60
SE	± 0.10	± 0.50	± 0.04	± 0.05	± 0.03	± 0.06	± 0.60	± 0.40	± 0.10	± 4.3	± 0.1	± 0.10
LSD (*P* = 0.05)	0.30	1.50	0.10	0.10	0.90	0.10	1.80	0.10	0.30	12.7	0.3	0.50

BRR, Brown rice rate; MRR, Milled rice rate; HRR, Head rice rate; GLAC, Grain length after cooking; ER, Elongation ratio; GL, Grain length; GW, Grain width; L/B, Length breadth ratio; VER, Volume expansion ratio; WUR, Water uptake ratio; ASV, Alkali spreading value; Amy, Amylose content.

Prominent DH lines from CRHR7 with high grain yield included CR7-7 (7 235 kg/hm^2), CR7-12 (7 042 kg/hm^2), CR7-5 (6 937 kg/hm^2), CR7-52 (6 817 kg/hm^2) and CR7-55 (6 727 kg/hm^2).

Grain quality analysis of doubled haploids

Eight DH lines from CRHR5 (CR5-6, CR5-10, CR5-19, CR5-49, CR5-61, CR5-85, CR5-105 and CR5-124) and CRHR7 (CR7-2, CR7-5, CR7-7, CR7-12, CR7-40, CR7-52, CR7-55 and CR7-68) with superior agronomic performance were analyzed for physico-chemical and grain quality characteristics (Table 3). The brown rice rate varied between 74.51% to 78.53% for DHs from CRHR5 and 73.54% to 79.13% for DHs from CRHR7. Likewise, the milled rice rate recorded for DH lines from CRHR5 ranged from 57.65% to 68.51% with a mean value of 66.27% whereas the same ranged from 59.27% to 69.52% with a mean value of 65.46% for DH lines from CRHR7. Alkali spreading value, which gives an idea for gelatinization temperature of rice grain, ranged within 4–7 in the DHs of both the hybrids. Amylose content is a major grain quality character as it indicates the volume expansion and water absorption during cooking (Deyner et al, 2001). The amylose content in the grains varied from 22.41% to 24.65% for DHs from CRHR5 and 22.31% to 24.63% for DHs from CRHR7. Similarly, the range of kernel length after cooking was 8.67–10.13 mm for DHs from CRHR5 and 8.66–11.23 mm for DHs from CRHR7. The ideal range of water uptake value was 145–195 in all the DHs. In addition, the volume expansion ratio of most of the DH lines fell within the acceptable range of 3.25–3.50 in both the hybrids. Overall, our results showed that three DHs from CRHR5 (CR5-10, CR5-49 and CR5-61) and four DHs from CRHR7 (CR7-5, CR7-7, CR7-12, and CR7-52) demonstrated superior grain quality characteristics such as head rice rate, alkali spreading values, grain length, volume expansion ratio and amylose content. Taken together with their high agronomic performance, these DH lines can be subjected to multi locational yield trial towards subsequent release as new high yielding DH rice varieties.

DISCUSSION

Although rice hybrids have superior grain yield in compared to conventional high yielding rice varieties, they are highly prone to segregation and lose their yield levels with subsequent generations. Doubled haploid

breeding is a novel approach towards development of new lines with retained qualities and characteristics. In the present study, we attempted to generate superior DH lines from two elite indica rice hybrids, CRHR5 and CRHR7. A significant difference in the anther culture response was found among the genotypes. This is consistent with the earlier report which suggests significant interaction among media, genotypes and their variable response to callus induction (Khalequzzaman et al, 2005; Bagheri and Jelodar, 2008). CRHR5 and CRHR7 were reported to have significantly higher callus induction with N6 and MO19 medium. An earlier experiment has also demonstrated significant callus induction in indica rice genotypes using N6 medium (Mishra et al, 2011). Although, CRHR5 and CRHR7 showed high callus induction (34.56% and 30.14%), the regeneration rates were low (21.12% and 19.56%) with high albinism (17.11% and 15.87%). This suggests that greater attention is needed to enhance the frequency of green plant regeneration. In addition, efforts are needed to minimize the albino plant production acting as a formidable obstacle to the utilization of rice anther culture for indica rice improvement. Shortening the culture period can be one of the strategies to reduce albinism.

The occurrence of around 40% doubled haploids in the anther derived progenies of indica rice can greatly help in breeding new varieties using anther culture. Of the four approaches employed (morphological, pollen fertility analysis, flow cytometry and cytology), the classical cytological approach through chromosome counts was ideal to determine the ploidy status. However, the approach is tedious and time consuming, and requires considerable expertise. The other cytology, based on determination the size of the pollen grain, an indirect method, has been utilized as an alternative convenient, rapid and reliable method to identify the ploidy level of many plant species (Zonneveld and van Iren, 2001). In the present study, flow cytometry was also used to precisely distinguish among the halploids, diploids, triploids and tetraploids. Flow cytometry is an attractive method for ploidy analyses as it is simple, easy and highly accurate (Ochatt, 2008). It is being frequently used to analyze the ploidy level of individuals obtained from the experiments of haplo-diploidization or chromosome doubling (Grewal et al, 2009; Ochatt et al, 2009) and to speed up the industrial exploitation of genotypes emerging from ploidy manipulation *in vitro* (Koutoulis et al, 2005).

The range of segregation among the DH lines from CRHR5 and CRHR7 as observed for all important agronomic traits like duration, plant height, panicle length, tiller number, yield, and grain characters was wide enough to select genotypes with desirable traits. Though the cultural response and the ploidy levels in the regenerations may depend on the genotype, the stability and uniformity displayed by the DHs appear to be genotype independent as 97%–98% of the DHs showed 100% uniformity and stability for all the characters in both the generations. The low variations observed within lines over generations support the presence of high levels of uniformity in the DHs. Similarly, the high level of synchrony in flowering among the tillers and plants of the DHs ensured uniform maturity of the crop. The stability and uniformity observed in the DHs are in agreement with the earlier reports (Suhartini and Hanarida, 2000; Sasmita, 2010). Of the 19 promising entries evaluated in the A_2 generation, a minor fraction of the DHs from each hybrid demonstrated yield levels at par with the parental values. This suggests that among the wide variation present in DHs lines that possess the desired traits required agronomic characters at the desired levels, i.e. duration, plant height, panicle length, tiller number, 1000-grain weight, and fertility could be selected. It was also observed that the uniformity of the traits in the same line as well as the high variability among different lines is an important characteristic of the breeding populations.

Rice breeders are more focused on grain quality improvement for high export value. Grain length, grain width and head rice rate are some of the important quality factors for rice processing industry (Kanchana et al, 2012). A selected set of DHs from CRHR5 and CRHR7 were also evaluated for quality characteristics. The rice cooking qualities characterized for DH lines included water uptake, grain length during cooking and volume expansion ratio. Majority of the DHs demonstrated superior grain quality and cooking characteristics. This suggests that the DH breeding retains not only the morpho-agronomic traits but also the physico-chemical qualities of the parental lines as desired in the DHs.

ACKNOWLEDGEMENTS

Authors are thankful to Director, Central Rice Research Institute, Cuttack, India for his support and guidance. Authors are also thankful to Dr. Ashutosh MALL (IGFRI, Jhansi) and Dr. N.N. JAMBHULKAR

(CRRI) for statistical analysis.

SUPPLEMENTAL DATA

The following materials are available in the online version of this article at http://www.sciencedirect.com/science/journal/16726308; http://www.ricescience.org.

Supplemental Table 1. Ploidy analysis of the regenerations derived from F_1 hybrid.

Supplemental Table 2. Analysis of variance of data on duration, plant height, panicle length, tiller number, 1000-grain weight and yield of the doubled haploids of CRHR5 in A_1 generation.

Supplemental Table 3. Analysis of variance of data on duration, plant height, panicle length, tiller number, 1000-grain weight and yield of the doubled haploids of CRHR5 in A_2 generation.

Supplemental Table 4. Analysis of variance of data on duration, plant height, panicle length, tiller number, 1000-grain weight and yield of the doubled haploids of CRHR7 in A_1 generation.

Supplemental Table 5. Analysis of variance of data on duration, plant height, panicle length, tiller number, 1000-grain weight and yield of the doubled haploids of CRHR7 in A_2 generation.

Supplemental Fig. 1. Phenotypic segregation pattern of the doubled haploid lines derived from CRHR5.

Supplemental Fig. 2. Phenotypic segregation pattern of the doubled haploid lines derived from CRHR7.

REFERENCES

Bagheri N, Jelodar N B. 2008. Combining ability and heritability of callus induction and green-plant regeneration in rice anther culture. *Biotechnoly*, **7**(2): 287–292.

Bhonsle S J, Sellappan K. 2010. Grain quality evaluation of traditionally cultivated rice varieties of Goa, India. *Rec Res Sci Techn*, **2**(6): 88–97.

Chu C C, Wang C C, Sun C S, Chen H, Yin K C, Chu C Y, Bi F Y. 1975. Establishment of an efficient medium for anther culture of rice through comparative experiments on the nitrogen sources. *Sci Sin*, **18**: 659–668.

Dewi I S, Purwoko B S, Aswidinnoor H, Somantri I H, Chozin M A. 2009. Plant regeneration from anther cultures of several genotypes of indica rice tolerant to aluminum toxicity. *Ind J Agric Sci*, **2**: 1–5.

Deyner K, Johnson P, Zeeman S, Smith A M. 2001. The control of amylase synthesis. *J Plant Physiol*, **158**: 479–487.

Feng Y, Zhai R R, Lin Z C, Cao L Y, Wei X H, Cheng S H. 2013. QTL analysis for yield traits in rice under two nitrogen levels. *Chin J Rice Sci*, **27**(6): 577–584. (in Chinese with English abstract)

Grewal R K, Lulsdorf M, Croser J, Ochatt S, Vandenberg A, Warkentin T D. 2009. Doubled-haploid production in chickpea (*Cicer arietinum* L.): Role of stress treatments. *Plant Cell Rep*, **28**: 1289–1299.

Herawati R, Purwoko B S, Dewi I S. 2010. Characterization of doubled haploid derived from anther culture for new type upland rice. *Ind J Agron*, **38**(3): 170–176.

INDOSTAT. 2004. Indostat Package for Data Analysis. Amearpet, Hyderabad, India.

IRRI. 2005. IRRISTAT for Windows: A Statistical Package for Analysis of Data. Manila, the Phillipines: International Rice Research Institute.

Kanchana S, Lakshmi Bharathi S, Ilamaran M, Singaravadivel K. 2012. Physical quality of selected rice varieties. *World J Agric Sci*, **8**: 468–472.

Khalequzzaman M, Haq N, Hoque M E, Aditya T L. 2005. Regeneration efficiency and genotypic effect of 15 indica type Bangladeshi rice (*Oryza sativa* L.) landraces. *Plant Tiss Cult*, **15**: 33–42.

Koutoulis A, Roy A T, Price A, Sherriff L, Leggett G. 2005. DNA ploidy level of colchicine treated hops (*Humulus lupulus* L.). *Sci Hort*, **105**: 263–268.

Lee J H, Lee S Y. 2002. Selection of stable mutants from cultured rice anthers treated with ethyl methane sulfonic acid. *Plant Cell Tiss Org Cult*, **71**: 165–167.

Mishra R, Rao G J N, Rao R N. 2013. Effect of cold pretreatment and phytohormones on anther culture efficiency of two indica rice (*Oryza sativa* L.) hybrids-Ajay and Rajalaxmi. *J Exp Biol Agric Sci*, **1**(2): 69–76.

Mishra R, Rao R N, Rao G J N. 2011. Anther culture response of indica rice hybrids. *Oryza*, **48**(4): 375–377.

Murashige T, Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant*, **15**: 473–497.

Niizeki H, Oono K. 1968. Induction of haploid rice plant from anther culture. *Proc Jap Acad*, **44**: 554–557.

Ochatt S J. 2008. Flow cytometry in plant breeding. *Cytom Part A*, **73**: 581–598.

Ochatt S J, Pech C, Grewal R, Conreux C, Lulsdorf M, Jacas L. 2009. Abiotic stress enhances androgenesis from isolated microspores of some legume species (Fabaceae). *J Plant Physiol*, **166**: 1314–1328.

Raina S K. 1989. Tissue culture in rice improvement: Status and potential. *Adv Agron*, **42**: 339–397.

Raina S K, Zapata F J. 1997. Enhanced anther culture efficiency of indica rice (*Oryza sativa* L.) through modification of the culture media. *Plant Breeding*, **116**: 305–315.

Ratheika V, Silva T D. 2007. A study on the anther culture response in different varieties of rice (*Oryza sativa* L.) subspecies indica. *In*: Proceedings of the 27th Annual Sessions of the Institute of Biology, Sri Lanka: 28.

Sasmita P. 2010. Evaluation of uniformity, variability, and stability of agronomic traits of doubled haploid rice lines resulting from

anther culture. *Bioscience*, **2**: 67–72.

Silva T D, Achala H H K. 2008. Microspore staging for haploid cell culture in *Oryza sativa* subspecies indica (rice). *In*: Proceedings of the 64th Annual Sessions, Sri Lanka: Page.

Suhartini T, Hanarida I. 2000. Genetic similarity of rice lines resulted from F-1 anther culture of H1 generation. *Penelitian Tanaman Pangan*, **19**(2): 13–20.

Zapata-Arias F J. 2003. Laboratory protocol for anther culture technique in rice. *In*: Maluszynski M, Kasha K J, Forster B P, Szarejko I. Doubled Haploid Production in Crop Plants. Dordrecht, Netherlands: Kluwer Academic Publishers: 109–116.

Zonneveld B J M, van Iren F. 2001. Genome size and pollen viability as taxonomic criteria: Application to the genus *Hosta*. *Plant Biol*, **3**: 176–185.