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Original Paper

Doubled Haploids generated through anther culture from an elite long duration rice hybrid, CRHR32: Method optimization and molecular characterization

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Abstract An improved procedure has been developed for high frequency androgenesis in an elite long duration *indica* rice hybrid. The effects of cold temperature pretreatment, duration of treatment and media with different plant growth regulators on callus induction and shoot regeneration were examined for generation of doubled haploids. N6 medium supplemented with 2.0 mgl^{-1} 2,4-D, 0.5 mgl^{-1} BAP and 30 gl^{-1} maltose was found to be most effective for callusing when compared with MS and SK1. The N6 media grown calli showed maximum green shoot regeneration frequency in MS medium supplemented with 0.5 mgl^{-1} NAA, 0.5 mgl^{-1} Kinetin, 1.5 mgl^{-1} BAP and 30 gl^{-1} sucrose after 2 week of culture. The cold temperature treatment of spike at 10°C for 2 days alongside by 8 days was found to be most suitable conditions for callusing and green shoot regeneration producing 186 green plants from *indica* rice hybrid, CRHR32. The ploidy status assessed on the basis of morpho-agronomic characters revealed fertile diploids at a frequency of about 81.10%; 16.10%, 2.68% and 1.08% were polyploids, haploids and mixploids respectively. Microsatellite marker analysis showed 1 : 1 ratio of the alleles of CMS and restorer lines used for development of CRHR32. Homozygosity was detected for all the marker loci in 150 DHs and only one plant was identified as heterozygote. This investigation identified the favorable media composition and condition for callus induction and green plant regeneration which would further increase the knowledge and better understanding in rice hybrids for development of DHs.

Key words: anther culture, doubled haploid, rice hybrid, segregation distortion, SSR.

Rice as a cereal grain is the predominant staple food for a large part of the world's human population, especially in Asia. It is estimated that by 2020 the rice consumers will be increased by two folds (Kabir et al. 2008) and world's population will reach eight billion by 2025 (Khush 2005). Therefore, increasing rice yield has become the most important goal of rice production on less land with limited resources. Since increase maximum amount yield in rice has become static after the green revolution, globally hybrid rice is considered as a best option showing significant yield advantages over inbreds in productivity and production per unit area. However, although a number of rice hybrids are released for commercial cultivation in India, still it does not gain its popularity among the Indian farmers for the reasons being: 1) high seed cost, 2) farmers cannot use their seed from one year to another, 3) unpredictable environmental condition and asynchronised flowering affects sterility expression during seed production which results in less seed production and 4) quality of the produce. Doubled haploid approach can effectively address the problems associated with hybrid rice through production of high yielding doubled haploids with uniform grain quality (Tariq et al. 2008). Moreover, this approach also has minimized some of these problems associated with hybrid rice in a successful run for last two decades within a short span of time using anther culture technique (Herath and Bandara 2011; Premvaranon et al. 2011).

Anther culture has been exploited to develop a number of varieties and improved breeding lines mostly in *japonica* cultivars (Grewal et al. 2011). However, adoption of this technique in *indica* cultivars is still limited due to their poor androgenic response (Dewi et al. 2009) for which attempts have been made to overcome the low anther culturability by evaluating a number of key factors involved in affecting the success of anther culture (Jacquard et al. 2006; Trejo-Tapia et al. 2002). Besides, there are other crucial problems such as anther necrosis, poor androgenesis frequencies and albino plant regeneration which hinder the successful application of anther culture technique(s) in *indica* rice

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(Gosal et al. 1997). Since to date, the reports are meager on DHs production in elite indica rice hybrids through anther culture, the present investigation was undertaken to optimize physical and chemical factors on callus induction and green plant regeneration for successful generation of doubled haploids from CRHR32, a long duration elite *indica* rice hybrid. CRHR32 is a rice hybrid developed first time in India which is suitable to address 10 million hectares underexploited irrigated shallow low land area of Eastern India. This has already shown its popularity by commercializing through eight private agencies which covers more than 20000 hectares area over three states such as Odisha, Bihar and Gujarat. Simultaneously, the parental allelic distribution in doubled haploids was assessed to confirm the origin either from pollen or somatic tissues of the F_1s through molecular analysis.

Materials and methods

Plant material

This study was conducted at the National Rice Research Institute (Formerly CRRI), Cuttack, Odisha, India. An elite long duration rice hybrid, CRHR32 developed from a cross between CRMS31A and CRL22R by NRRI was taken for the study. Thirty day old seedlings, raised in dry seed beds under ideal conditions, were transplanted in well puddled field with spacing of 20 cm×15 cm between and within rows. Recommended doses of N : P : K (100 : 50 : 50) were applied in three split doses and need based crop protection measures were undertaken. Two tests were carried out in different years during June to October, 2012 and 2013. Spikes for both tests were collected in September at varying stages of emergence to secure sufficient numbers of anthers with microspores at the desired developmental stages. Panicles at the booting stage, 8–12 cm in length, that were positioned between the subtending leaf and the flag leaf were collected.

Boots collection and cold pretreatment

Boots from primary and secondary tillers were sampled at appropriate stage in the morning (7AM–8AM) from the field grown plants. Then the boots were wiped clean 2–3 times, with a clean muslin cloth moistened with 70% alcohol, wrapped in wet non absorbent cotton and were enclosed inside polythene bags in order to prevent desiccation and maintain pollen viability. The wrapped boots were placed in an incubator that was maintained at different temperatures (4°C, 8°C, 10°C, 12°C) for 2, 4, 6, 8, 10, 12 days in darkness for cold pretreatment. Control spikes were not subjected to cold treatment. The materials for cold pretreatment was prepared by keeping boots intact with their penultimate leaf sheath and node, and trimming off flag leaf and extra basal nodes. Most of the boots then harvested when the growth of anthers attained around half (1/2) to one third (1/3) of spikelet length. The middle portion of the panicle from the pretreated spikelet was used for anther culturing. The boots were surface sterilized with 70% ethanol for 4 min followed by 4% commercial bleach (NaOCl) for 2 min and rinsed three times with sterile de-ionized water. Before culturing of anthers from a hybrid, the anthers with mid to late uninucleate stages were first determined by cytological test of microspore using acetocarmine staining and 40–50 anthers were uniformly dusted over the surface of the media. The cytological test result was later traced back with anthers position on spikelet.

Culture media and condition

For callus induction, three different basal media i.e. MS (Murashige and Skoog 1962), N6 (Chu et al. 1975) and SK1 (Raina and Zapata 1997) were prepared with different combinations and concentrations of BAP (0.25–1.0 mg l^{-1}), Kinetin (1.0 mg l^{-1}) and 2,4-D (1.5–2.5 mg l^{-1}) along with 30 g l^{-1} maltose (Table 1). For shoot regeneration, two media were formulated using MS medium along with combinations of growth regulators (BAP, Kn and NAA) and 30 g l^{-1} sucrose. Likewise, MS media supplemented with NAA (2.0 mg l^{-1}), Kn (0.5 mg l^{-1}) and 50 g l^{-1} sucrose were used for rooting. The pH of all the media was adjusted to 5.8 using 0.1 N NaOH/0.1 N HCl and solidified with 8 g l^{-1} agar-agar (Himedia, India) before autoclaving. Routinely, 25 ml of molten media was dispensed in to culture tubes (25×150 mm), plugged with non-absorbent cotton wrapped in one layer of cheesecloth and sterilized at 121°C (15psi) for 15 min. The cultured anthers were incubated in dark at 25±2°C for callus induction while all other cultures were incubated at temperature of 25±2°C with 42 μmol m^{-2}/s^{-1} illumination (cool, white fluorescent lamps) under 16 h photoperiod. The green plantlets when they reached a size of around 1 cm in length were transferred to rooting medium for root formation.

Acclimatization and net house transfer

The resulting green regenerants with well-formed roots were removed from agar and transplanted to pots containing sterile

Table 1. Basal media supplemented with growth regulators for callus induction from anthers of CRHR32

Basal Media+Growth regulators (mg l^{-1})					
N_6		MS		SK1	
N_1	N6+2.0 2,4-D+0.1 Kn	M_1	MS+2.0 2,4-D+0.1 Kn	S_1	SK1+2.0 2,4-D+0.1 Kn
N_2	N6+2.0 2,4-D+0.5 BAP	M_2	MS+2.0 2,4-D+0.5 BAP	S_2	SK1+2.0 2,4-D+0.5 BAP
N_3	N6+2.5 2,4-D+1.0 BAP	M_3	MS+2.5 2,4-D+1.0 BAP	S_3	SK1+2.5 2,4-D+1.0 BAP
N_4	N6+1.5 2,4-D+0.25 BAP	M_4	MS+1.5 2,4-D+0.25 BAP	S_4	SK1+1.5 2,4-D+0.25 BAP

soil; the pots were kept in shade for 3–4 days. Thereafter, the plants were transferred to net house and necessary culture management was undertaken for good growth and development of the plant.

Ploidy evaluation

The plants were evaluated for their ploidy status on the basis of morphological characters. Plants with normal morphological appearance and fertility were considered as diploids. Haploid plants are supposed to be small and sterile while tetraploids or polyploids showed tall, gigantic growth along with thick dark green leaves. Besides, the mixploids were identified after visualizing the grain characters in individual tillers.

SSR analysis

All the plants found to be fertile diploids were differentiated to distinguish the heterozygotes (donor like) and doubled haploids using SSR markers and also to determine their source of origin from either parents used for development of rice hybrid. A total of 650 SSR markers were screened out of which 40 pairs of microsatellite rice markers showing heterozygosity or parental polymorphism were used. Total genomic DNA was extracted from young leaves by modified CTAB method (Murray and Thompson, 1980). Amplification reactions were performed in volumes of 10 μl containing 1 X PCR buffer (Biotool B&M Labs, Spain) (75 mM Tris HCl; pH 9.0), 50 mM KCl, 20 mM $(NH4)_2SO_4$, 2 mM $MgCl_2$, 0.25 mM dNTPs (MBI Ferment Inc., Maryland, USA), 10 pmole of each of the primers, 1.0 unit of *Taq* DNA polymerase (Biotool B&M Labs, Spain) and 25 ng of template DNA. The amplification reaction was carried out in a DNA Thermal Cycler (Eppendorf AG, Hamburg, Germany) programmed for 36 cycles as follows: 1st cycle of 4 min at 94°C followed by 35 cycles each of 30 s denaturation at 94°C, 45 s annealing at 55°C and 1 min extension at 72°C. The final step consisted of one cycle of 10 min at 72°C for complete polymerization. After completion of the PCR, 2.5 μl of 6X loading dye (MBI Ferment Inc., Maryland, USA) was added to the amplified products and were electrophorized in a 2.5% (m/v) agarose (Bangalore Genei, Bangalore, India) gels with 1X TBE buffer, stained with ethidium bromide and documented by a gel documentation system (Syngene, Cambridge, UK). The size of amplification products was estimated by comparing with standard DNA ladder (O'Gene Ruler 100 bp DNA ladder; MBI Ferment Inc., Maryland, USA). All the reactions were repeated three times. All the primers were amplified on the basis of the clarity of the banding patterns.

Observation of cultures and statistical analysis

Each treatment had 20 cultures and each experiment was repeated at least three times. The frequency of callus induction (CI %) was estimated as the number of anthers producing calli (approximately 0.5–2.0 mm diam.) per number of anthers cultured. Callus induction frequency was recorded after 3–4 weeks of culture. Data on the percentage of calli regenerating green and/or albino shoots were recorded after 2–4 weeks culture. The frequency of callus induction and shoot regeneration was calculated as follows:

Callus induction frequency (%):

$$\frac{\text{Number of anthers producing calli}}{\text{Number of anthers cultured}} \times 100$$

Shoot regeneration frequency (%):

$$\frac{\text{Number of shoots recovered}}{\text{Number of calli cultured}} \times 100$$

The data pertaining to mean culture percentage of CI, percentage response of callus for shoot regeneration, percentage of green and albino shoot regeneration were statistically analysed using analysis of variance (ANOVA) and differences among means were compared by the Duncan's Multiple Range Test (DMRT) in SAS software (SAS 9.1; SAS Institute, 2003). Between the treatments, the mean having the same letter are not significantly different at the $p<0.05$ level.

Results

Effects of cold temperature, duration of treatment and media with PGRs on callus induction

Anthers containing pollen at the mid uninucleate stage of development were used for culture (Figure 1A). A preliminary study was undertaken to evaluate the callus induction potential in various temperatures (4, 8, 10 and 12°C) for a period of 0–12 days from which it was observed that the 10°C temperature was found to be best irrespective of all the media (Table 1) and period of incubation tested (Data not shown). There is no sign of callusing in the anthers pretreated at 4°C, 8°C and 12°C cultured for a prolonged period. Anthers of suitable stages were cultured on N6, MS and SK1 medium with different combinations and concentrations of growth regulators. After 21 days of incubation at 25±2°C in total dark, anthers were swollen and turned brown and callus induction started, which continued up to 45 days. The anthers first turned brownish, swollen and subsequently calli emerged asynchronously after 3–4 weeks of culture in N6 medium and after 6–7 weeks in MS and SK1. The anther derived calli showed varied textures and colours in different nutrient media. Compact or friable, soft and watery texture with different colors such as yellowish, white, and transparent calli were observed. Yellowish and compact calli were found in N6 and MS media while whitish, transparent, soft, watery and friable calli was observed only in SK1.

Of the pretreatment periods (0–12 day) at 2 day interval evaluated, callus induction was observed to be significantly high when the spikes were pretreated at 10°C for 2 day followed by 8 day as compared to other days (0, 4, 6, 10 and 12 day) (Table 2). There was no sign of callus induction from the anthers on 4, 6 day pretreated spikes in N6 media, 4, 6 and 12 day in MS

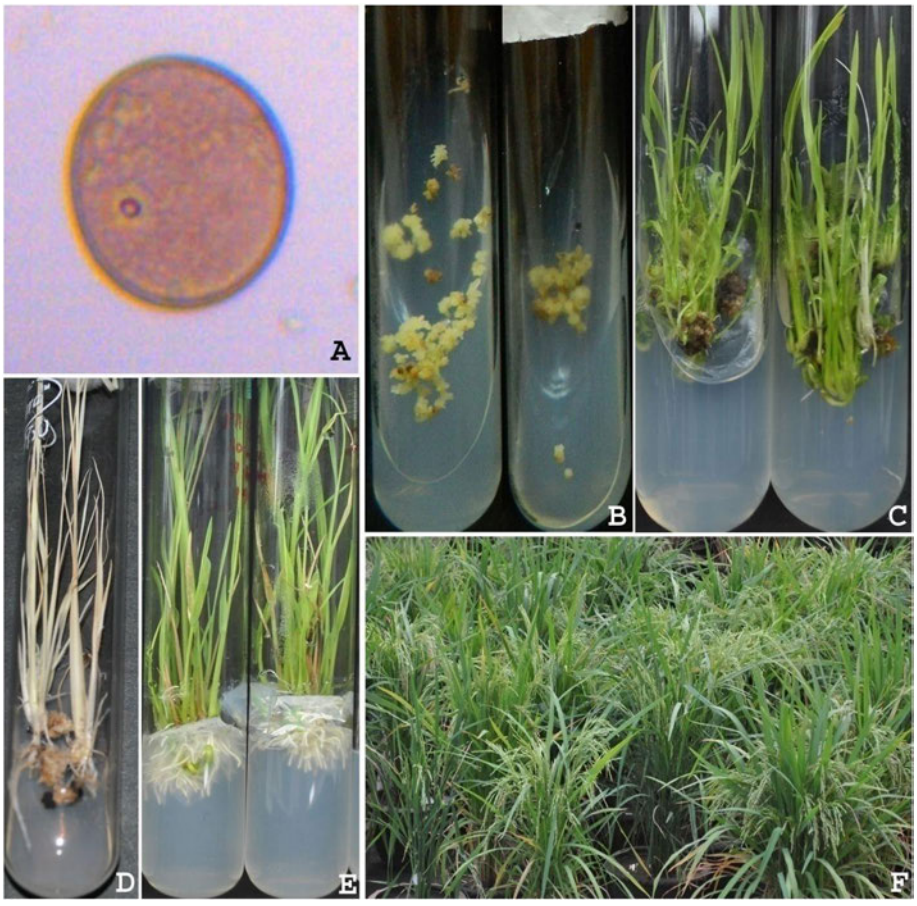

Figure 1. Development of plants via callus culture from CRHR32 through androgenesis (A) Mid uninucleate stage of microspore (bar 5 mm), (B) Calli induction from pollens of anthers (bar 10 mm), (C) Green shoot regeneration from calli (bar 10 mm), (D) Albino generated from calli (bar 5 mm), (E) Rooting in microshoots (bar 10 mm), (F) Anther derived plants grown in net house (bar 0.5 cm).

Table 2. Effect of 10°C incubation and period of pretreatments on response of calli induction in N6, MS, SK1 medium with different growth regulators from anthers of CRHR32

N6 Medium	Duration of cold pretreatment (day) Callus induction (%)			MS Medium	Duration of cold pretreatment (day) Callus induction (%)			SK1 Medium	Duration of cold pretreatment (day) Callus induction (%)	
	2nd	8th	10th		2nd	8th	10th		2nd	8th
N1	3.89c	5.76b	1.94bc	M1	3.83b	1.37b	1.94c	S1	2.43a	0.06b
N2	16.35a	9.25a	5.39a	M2	6.73a	5.61a	5.39a	S2	0.05c	0.80a
N3	5.29b	3.99b	2.63b	M3	3.68b	2.30b	2.63b	S3	0.80b	0
N4	3.93c	1.33c	1.17c	M4	2.43b	1.59b	1.17c	S4	1.04b	0

Means having the same letter within a column were not significantly different by DMRT test at $p<0.05$. Each treatment was replicated three times; each replicate consisted of 20 cultures.

media and 4, 6, 10 and 12 day in SK1 media after 32-week of culture. No response for callus induction was observed in the anthers devoid of cold temperature pretreatment. BAP in combination with 2,4-D was found to be most favorable in inducing calli whereas Kn showed low response for callus induction. There was no sign of callusing when anthers were cultured in media without cytokinin and auxin. The response of calli was found to be low (3.93%) in N6 supplemented with 0.25 mg l^{-1} BAP and 1.5 mg l^{-1} 2,4-D (N4). However, increased concentration of BAP by two times and 2,4-D by 1.3 times in N2 enhanced 2.5 times callus induction frequency in N_6 culture medium (Table 2; Figure 1B). At higher concentration and combinations of BAP and 2,4-D (N3), the rate of callus induction declined. The calli induction frequency varied from 3.89–16.35% and 1.33–9.25% in 2 day and 8 day incubation respectively in N6 media. Callus induction was significantly high in the spikes pretreated for 2 days at 10°C on N6 media supplemented with BAP (0.5 mg l^{-1}) and 2,4-

Table 3. Response of shoot regeneration in MS medium with different growth regulators from the calli induced in N6, MS medium in CRHR32

MS medium+Growth regulators ($mg\,l^{-1}$)	Duration of cold pretreatment (day) Shoot regeneration (%) from N6 CI media						Duration of cold pretreatment (day) Shoot regeneration (%) from MS CI media					
	2nd		8th		10th		2nd		8th		10th	
	G	A	G	A	G	A	G	A	G	A	G	A
1.5 BAP+0.5 Kn+0.5 NAA	71.66a	3.84b	42.17	9.44a	32.89a	10.94b	25.96a	3.73	8.52a	1.45	7.27	1.59
0.5 BAP+1.5 Kn+0.5 NAA	48.13b	13.15a	35.03	6.22b	25.44b	14.50a	14.63b	4.44	2.48b	1.81	3.35	1.36
LSD at 5%	6.86	2.91	NS	1.65	4.20	1.47	4.809	NS	3.35	NS	NS	NS

Means having the same letter within a column were not significantly different by DMRT test at $p<0.05$; Each treatment was replicated three times; each replicate consisted of 20 cultures. NS, non significant; G, Green; A, Albino; CI, callus induction media.

D ($2.0\,mg\,l^{-1}$) while in case of MS medium (M2) the response was 2.5 times lower than the N6 media; very low callus response was found in SK1 (S2). A cold temperature pretreatment at 10°C for 2 day yielded the best callus induction followed by 8 day for the F_1 hybrid, CRHR32. The colour of the calli derived from 10°C pretreated anthers was yellowish, compact and non-friable in N6 and MS media while whitish, friable calli was observed only in SK1. The overall frequency (16.35%) of callus induction on Chu's N6 media was found better than that of MS media, showing the superiority of N6 media over the MS for the induction of callus (Table 2). The callusing ability was maintained up to 8-week period on N6 $+0.5\,mg\,l^{-1}$ BAP $+2.0\,mg\,l^{-1}$ 2,4-D $+30\,g\,l^{-1}$ maltose. The calli induced in SK1 showed necrosis which eventually died.

Effects of media composition and duration of treatment on shoot regeneration

Another study was conducted to see the effects of basal media and growth regulators on shoot regeneration from the calli induced in N6 and MS media; these calli were developed from the spikes pretreated with different durations at 10°C. The calli of 1–2 mm in size emerging from the cultured anthers were transferred to regeneration medium. Within two weeks of culture, the transferred calli started differentiating into clumps of green spots and then into green shoots (Figure 1C). Some of the calli, instead of forming green spots exhibited white shoot like structures which subsequently developed albino plants (Figure 1D). It was observed that the 2 day pre incubated calli grown in N_6 basal media cultured in to MS basal media showed significantly higher green shoot regeneration followed by 8 day pre treatments at 10°C (Table 3). Similar trend was found in shoot regeneration for the calli grown in MS media for pretreatment periods. However, highest green shoot regeneration (71.66%) was observed in MS+BAP $(1.5\,mg\,l^{-1})$+Kinetin $(0.5\,mg\,l^{-1})$+NAA $(0.5\,mg\,l^{-1})+30\,g\,l^{-1}$ sucrose from the calli induced in N6 medium. Higher concentration $(1.5\,mg\,l^{-1})$ of BAP along with low concentration of Kinetin $(0.5\,mg\,l^{-1})$ was found more effective for shoot regeneration than high Kinetin $(1.5\,mg\,l^{-1})$ and low BAP $(0.5\,mg\,l^{-1})$ in MS media (Table 3). The overall study showed that calli grown in N6 media was found to be more effective for green shoot regeneration in MS media. The green shoots varied from 48.13–71.66% and 35.03–42.17% in MS1 and MS2 from the 2 day and 8 day pretreated anther derived calli respectively grown in N6 media. The frequency of albino plants developed was low as compared to the frequency of green plants in all the three media tested. Remarkable differences were observed (Table 3) between callus induction medium-regeneration medium combinations when calli induced on a particular basal medium were regenerated on a different basal medium.

Out of 220 calli, 186 green shoots differentiated from individual calli while 10 produced only albinos; rest of the calli showed necrosis. Only the well grown green shoots were further transferred into rooting media. A total of 186 micro green shoots formed a high percentage (100%) of roots grown in MS media supplemented with $2.0\,mg\,l^{-1}$ NAA $+0.5\,mg\,l^{-1}$ Kinetin $+50\,g\,l^{-1}$ sucrose (Figure 1E). Well developed plants with profuse roots were acclimatized and transferred phase by phase to the net house and survived showing promising growth from which a number of plants showed variability in terms of height, panicle size and grain types (Figure 1F). The height of anther derived plants varied from 101.0–121.4 cm while the donor (CRHR32) showed height of 118.0 cm. Similarly, CRHR32 produced the panicle length of 28.9 cm whereas the panicle length of the anther derived plants ranged from 23.3–29.2 cm.

Ploidy of regenerated plants

Ploidy levels of the anther derived plants were confirmed by studying the morphological characters. Out of 186 green plants, 151 showed normal morphological appearance with 65–77% grain fertility, 30 plants were polyploids with tall, large and broad thick leaves with less than 1% of spikelet fertility and 5 plants showed short stature with no spikelet fertility thus confirming as haploids (Figure 2A). Only 2 plants were found to be mixploids which were discriminated by observing the grain type in the panicles after the grain maturity.

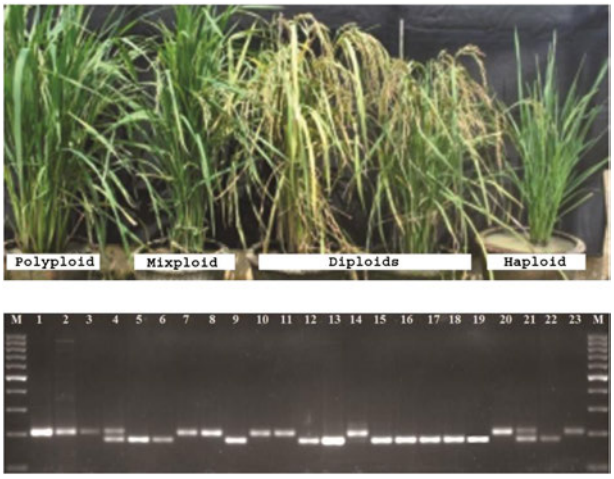

Figure 2. Ploidy differentiation in anther derived plants of CRHR32 (A) Agro-morphological characters distinguishing polyploidy, mixploid, diploid and haploid plants (bar 5 cm) and (B) SSR marker (RM480) differentiating somatic tissue derived heterozygote from doubled haploids (1–3 & 5–20: doubled haploids; 4: heterozygote (arrow); 21: CRHR32; 22: CMS31A; 23: CRL22R; M: 100 bp DNA ladder.

Parental allelic distribution and identification of somatic tissue derived diploids using SSR analysis

A total of 650 SSR markers were screened to survey the parental polymorphism out of which 40 showed polymorphism (6.15%) between CRMS 31A and CRL 22R. Further, all 40 markers were used in characterizing 151 DH lines generated from CRHR 32. Simple sequence repeat analysis revealed homogeneity for 99.34% of the total marker evaluated in 151 DH lines. Out of these lines, 150 showed complete homogeneity for alleles of either parent, suggesting that DH lines are true representatives of the gametic constitution and are derived from the F1 pollen. However, variation was detected among 1 line that is heterozygous for the parental alleles which might be generated from somatic tissues (Figure 2B).

In order to check the segregation distortion, 40 polymorphic SSR markers amplified 80 alleles from 150 DHs derived from CRHR32 (Table 4); 53.12% and 46.88% of the alleles were found to be of CRMS31A and CRL22R type respectively. Out of 40 markers, 9 markers from seven chromosomes (22.5%) were found to be distorted from the expected genotypic frequencies (1 : 1). Besides, out of 9 distorted markers, 5 markers such as RM10992 and RM490 from chromosome 1, RM545 from chromosome 3, RM13 from chromosome 5, RM23744 from chromosome 9 showed significant distortion at $p<0.05$. While remaining four markers, RM13562 from chromosome 2, RM17111 and RM17008 from chromosome 4 and RM21559 from chromosome 6 were found to be deviated at $p<0.01$. Of total 18 alleles amplified from 9 distorted SSR markers, 72% alleles favoured for restorer parent (CRL22R) while the rest skewed to CMS line (CMS21A). However, 31 markers skewed favoring parental alleles which revealed the expected 1 : 1 ratio for the alleles of CRMS 31A and CRL 22R used for development of CRHR32.

Discussion

Anther culture technique is highly effective on better development of various important crops including rice (Chang and Coe 2009). Anther culture is of two step processes: the first step involves the induction of organogenic/ embryogenic calli from microspores and the next step deals with the regeneration of green plants from the calli (Bishnoi et al. 2000). Haploid microspores in anther can develop into callus and green plant regeneration if they are cultured in a good condition and at a selective stage of development and growth of gametophyte cells. In the present study, cold pretreatment, incubation time, different media combinations along with growth regulators were optimized for callus induction and green shoot regeneration in CRHR32, a rice hybrid variety.

Since temperature is one of the most important factors that influences the induction of callus development (Sopory and Munshi 1996), the type, duration and the time of application of pre-treatment vary with species or variety (Datta 2001). Our result showed that the cold pretreatment of spike showed a positive influence on the callus induction frequency irrespective of all the media tested. Though no incubation of spikes shows any sign of callus induction (Sen et al. 2011), surprisingly 2 day spike incubation at 10°C resulted in the best callus induction in the F_1s of CRHR32 at the mid-uninucleate stage which corroborated to the earlier finding of Genovesi and Magill (1979). The second highest calli induction was found in 8 day preservation time. Zapata-Arias (2003) recommended a treatment at 8–10°C for 8 days for callus induction in rice. The extended temperature and periods were found to be inhibitory for callus induction which is in agreement with earlier study (Lenka and Reddy 1994).

Besides the importance of incubation period, the basal media like MS, N6 and SK1 media play very significant role in optimization of callus induction. Inclusion of growth regulators like 2,4-D, BAP combinations into each medium were found to be effective on callus induction. The result showed that N6 medium supplemented with 2.0 mg l^{-1} 2,4-D, 0.5 mg l^{-1} BAP and 30 g l^{-1} maltose had the highest frequency of callus production which almost doubled up the production of callus as compared to MS with similar composition. Thus, the outcome of incorporating three basal media (N6, MS and SK1) with two growth regulators was not significant in callus induction. The overall frequency (%) of callus induction on Chu's N6 media was found to be best as compared to MS and SK1 media, showing

Table 4. SSR analysis showing allelic frequencies of parental lines in 150 doubled haploids of CRHR 32 using χ^2 test

No.	Chromosome no.	SSR Marker	SSR repeat motif	DH population (1 : 1 expected ratio)		
				F_a	F_b	χ^2-test
1	1	RM529	(CT)12	0.50	0.50	0
2	1	RM495[(1)]	(CTG)7	0.49	0.51	0.01
3	1	RM10992[(1)]	(AT)10	0.25	0.75	5.43*
4	1	RM490[(1)]	(CT)13	0.21	0.79	6.54*
5	2	RM71	(ATT)10T(ATT)4	0.50	0.50	0
6	2	RM279[(1)]	(GA)16	0.35	0.65	2.05
7	2	RM211[(1)]	(TC)3A(TC)18	0.39	0.61	3.24
8	2	RM13562[(1)]	(CT)10	0.20	0.80	7.34**
9	3	RM545[(1)]	(GA)30	0.25	0.75	5.67*
10	3	RM520[(2)]	(AG)10	0.54	0.46	0.32
11	3	RM3117[(1)]	(CA)12	0.31	0.69	2.87
12	4	RM16284[(2)]	(AT)28	0.52	0.48	0.04
13	4	RM280[(2)]	(GA)16	0.65	0.35	2.71
14	4	RM17111[(2)]	(AG)28	0.85	0.15	9.8**
15	4	RM17008[(1)]	(GA)26	0.1	0.90	12.8**
16	5	RM13[(1)]	(GA)6-(GA)16	0.29	0.71	5.87*
17	5	RM480 [(1)]	(AC)30	0.45	0.55	0.21
18	5	RM163[(1)]	(AG)15	0.44	0.56	0.32
19	6	RM584	(CT)14	0.50	0.50	0
20	6	RM204[(1)]	(CT)44	0.38	0.62	3.15
21	6	RM19422[(2)]	(AG)45	0.52	0.48	0.08
22	7	RM21559[(1)]	(AG)12	0.14	0.86	8.75**
23	7	RM336[(1)]	(CTT)18	0.30	0.70	3.03
24	7	RM481[(2)]	(CAA)12	0.62	0.38	2.52
25	8	RM23076[(1)]	(AG)24	0.39	0.61	3.21
26	8	RM22633[(2)]	(CT)16	0.60	0.40	1.67
27	8	RM22252	(CCG)10	0.50	0.50	0
28	9	RM205[(2)]	(CT)25	0.55	0.45	0.43
29	9	RM219[(1)]	(CT)17	0.44	0.56	0.03
30	9	RM316[(1)]	(AC)10	0.36	0.64	2.40
31	9	RM23744[(1)]	(CT)37	0.21	0.79	6.13*
32	10	RM25735[(1)]	(AAG)25	0.30	0.70	2.12
33	10	RM216[(1)]	(CT)18	0.46	0.54	0.13
34	10	RM25712[(2)]	(AG)12	0.55	0.45	0.47
35	11	RM26269	(TG)11	0.50	0.50	0
36	11	RM26652	(TTC)9	0.50	0.50	0
37	11	RM224[(2)]	(AAG)7	0.57	0.43	0.83
38	12	RM27574[(1)]	(AAT)23	0.40	0.60	2.11
39	12	RM28424	(ATA)34	0.50	0.50	0
40	12	RM28157	(TTA)36	0.50	0.50	0

[(1)] Markers segregated favoring CMS31A alleles, [(2)] Markers segregated favoring CRL22R alleles, * significantly deviated at 0.05 (χ^2=3.84 for DH), ** significantly deviated at 0.01 (χ^2=6.64 for DH), (F_a) frequency of A allele, (F_b) frequency of B allele.

superiority of N6 media over other two media used for callus induction. This might be due to the lower level of nitrogen in N6 as compared to MS and SK1. Lentini et al (1995) demonstrated the beneficial effects of lowered NH4+ levels on callus induction in *indica* rice. In addition to basal media, the plant growth regulators are also equally important in calli induction. Though a fairly high concentration of 2,4-D (2 mg l^{-1}) alone produced relatively high rate of callus induction in some genotypes (Raina and Zapata 1997), however, combination of low concentration (0.5 mg l^{-1}) of BAP with 2,4-D enhanced the rate of calli response considerably in our study. Moreover, BAP in combination with 2,4-D was found most favorable in inducing calli whereas Kn showed very low response for callus induction (Bohorova et al. 1995). It might be the synergistic effect of these two PGs used for callus induction.

A number of factors such as culture medium, growth regulators, culture environment, explant and genotype of donor are known to influence the callus induction and shoot regeneration. Usually, androgenesis aims at increasing the frequency of green plant regeneration from cultured anthers or microspores. However, this could be achieved by parallel improvement of the two phases of the anther culture process, callus induction and shoot regeneration. Our study showed the improved frequencies of callus production and green shoot regeneration from an earlier report where

callus induction and shoot regeneration frequencies in four indica hybrids were found 0–5.06% and 0–14.67% respectively (Sen et al. 2011).

In the present experiment, the shoot regeneration from calli was distinctly better in MS medium which might be due to higher concentration of NH4+ in the MS media as compared to N6 media. This finding is corroborated with Mandal and Gupta (1995) who reported that MS is better than N6 on shoot regeneration in anthers of *indica* rice. Besides, though plant growth regulators play an important role in green shoot regeneration, their effects on green plant production has been analyzed to a lesser extent than in the case of callus induction. In this study, inclusion of BAP, Kn in combination with NAA adequately supported green plant regeneration from sub-cultured callus which is in agreement with Bishnoi et al. (2000). Additionally, 2 day spike pretreatment at 10°C resulted in best shoot regeneration from the anther derived calli grown in N6 media. This finding was supported by Roininen et al. (2005) who observed that the cold pre treated spikes for shorter period increased green plant regeneration in anther culture. The result of the present study showed that cold stress could be effective for the improvement of *indica* rice anther culture. Different reasons have been suggested for the positive effect of cold pretreatment (Shahvali-Kohshour et al. 2013; Shariatpanahi et al. 2006). Cold pretreatment protects microspore from toxic compounds released in decaying anthers because it slows down degradation processes in the anther tissues. It induces starvation of microspores disconnected from the tapetum in cold-treated anthers. In addition, cold increases the total content of free amino acids, which might be conducive to an adaptation of microspores to the metabolic changes for androgenesis. In cold pretreatment, some small heat shock protein (HSP) genes could be expressed possibly to protect cells against chilling injuries. Moreover, the calli texture and colour also had some influence on androgenesis. The compact calii with yellowish color resulted in better green plant regeneration whereas soft calli with white color proliferated and resulted in callus multiplication with more albino plant formation.

The occurrence of a large proportion of albinos among the regenerated plants following anther culture is the most frustrating feature of androgenesis and remains a formidable obstacle in rice anther culture (Grewal et al. 2009; Khatun et al. 2012) especially in *indica* rice. In this study, 2 day spike incubation at 10°C and the anther derived calli cultured only for 10–12 day reduced the frequency of albinos compared to incubation at more than 10 days which is corroborated with the earlier reports (Asaduzzaman et al. 2003). Similarly, Chaleff and Stolarz (1981) reported better anther culture response in rice when anthers were pretreated at 7°C for 3-d in comparison to untreated one. Cold pretreatment is reported to enhance the blockage of the gametophytic development of microspores and guide the continuous division of the microspores into forming callus/embryo, a sporophyte during culture (Heberle-Bors et al. 1996); this mode of shifting from gametophytic to sporophytic may cause instability in microspore development resulting in loss of chlorophyll (Mishra et al. 2013).

Androgenetic produced plant population might show an increased segregation distortion due to the preferential regeneration of gametes coming from the better responding parent. Molecular analysis revealed that of the 151 fertile diploid lines, 150 originated from the pollen and one from somatic tissue of the F_1s. Since flow cytometer cannot distinguish somatic tissue derived diploids from DHs, the application of SSR markers successfully provided the best information on heterozygosity of the allelic loci for all the 151 diploid lines derived through anther culture (Grewal et al. 2011; Lapitan et al. 2009). Only 150 homozygous DH lines were used for further studies. A total 80 alleles characterized from 150 DH lines indicated that an equal amount of genetic material from each parent has been transmitted to the progenies through anther culture, which is evident from the even distribution of CRL 22R and CRMS 31A alleles in the DH population. The segregating markers in this study were in accordance with a 1 : 1 ratio at $p<0.01$.

Segregation distortion is a common phenomenon in anther culture-derived DHs (Graner et al. 1991; Heun et al. 1991; Thompson et al. 1991; Zivy et al. 1992) which can be analyzed using wide range of different markers such as morphological, isozymes and DNA markers (Zamir and Tadmor 1986). Of all the markers tested, DNA marker like SSR is widely adopted due to its co-dominant nature and abundant in rice genome. In the present study, out of forty polymorphic markers, 9 (22.5%) showed skewed segregation in DH population derived from anthers of CRHR32 which is found lower as compared to the DHs derived from *indica–japonica* inter-crossing (41%) (Huang et al. 1997). The presence of segregation distortion factors like hybrid sterility (S) genes and gametophyte competition (ga) genes are often associated with the segregation distortion in rice (Nakagahra 1972; Sano 1990). Besides, segregation distortion occurred more frequently in rice DH populations as compared to F2 populations (Xu et al. 1997). Segregation of DH lines with 72% alleles specific to CRL22R genotype might show an increased segregation distortion due to the preferential regeneration of gametes coming from the fertile parent as compared to the CMS parental line. Moreover, SD has been frequently observed to be in favour of the alleles of the parent performing superior in in vitro culture (Foisset and Delourme 1996). Therefore, superior alleles

from one of the best performing parental line might be linked/ associated with the genes controlling the ability to generate fertile plants and can be preferentially transmitted by anther culture processes.

The method established here is different from those published previously in certain key points: (a) 2 day incubation at 10°C of spike produced a fairy frequency of callusing and green shoot regeneration in *indica* rice; (b) anthers responded for callusing produced fairly significant frequency of green shoot regeneration in comparison to earlier report; (c) distorted segregation was found to be low in the DHs derived through anther culture.

In conclusion, this method for anther culture was developed and optimized to produce DH lines for an elite rice hybrid, CRHR32 manipulating the physical and media components. This method will certainly help other researchers to move fast from the in vitro method to large scale field trials, thereby accomplishing the field assessed DHs in a short period of time as compared to traditional breeding. These results also emphasized the importance of SSR markers for identification of somatic tissue derived diploids which would segregate in future. Besides, alleles of either parent assessed through SSR markers, suggest that DH lines are true representatives of the gametic constitution and are derived from the F1 pollen of CRHR32. In this study, though the green plant regeneration obtained are reasonably on the higher side, search for universally usable callus induction media for *indica* rice is still required for strengthening the DH approach through anther culture.

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