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DNA polymorphism among rice somaclones

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Abstract

Molecular markers were used to detect the influence of high concentrations of 2,4 dichlorophenoxyacetic acid (2,4-D) in the callusing media on DNA variations in regenerated rice plants. Restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD) and polymerase chain reaction (PCR) based RFLP analysis were carried out on 12 somaclones of *Oryza sativa* L. cv. B 370. *In vitro* culture induced DNA variations were detected in the regenerated plants but the effect of high auxin concentration in the medium could not be revealed. In a second study, fingerprinting of 15 semi-dwarf, high yielding somaclones of B-370 was carried out using RAPD technique. Amplification using 20 random primers produced a total of 167 DNA bands out of which 97 bands were polymorphic. A total of 32 unique DNA bands were detected across all the somaclones and they could be grouped based on their similarity to B-370. RAPD analysis helped to reveal similarity or differences among the somaclones while fingerprinting using additional RAPD markers was not successful.

Key words: 2,4-D, *Oryza sativa*, RFLP, RAPD, PCR based RFLP.

Introduction

The traditional *basmati* rice cultivars, such as B-370 and Karnal Local, are the most widely grown *basmati* cultivars in India. Strategies to improve these rice cultivars using *in vitro* techniques are in progress by many workers. However, *in vitro* culture of plant tissues has been found to induce an uncontrolled genetic instability (chromosomal aberrations, activation of transposable elements, methylation changes, amplification and deletion of genes, *etc.*: Phillips *et al.* 1994, Kaeppler *et al.* 1993,

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Peschke and Phillips 1992, Karp 1991, Muller *et al.* 1990, Rode *et al.* 1987). Among the factors affecting *in vitro* induced somaclonal variation, the use of phytohormones appears to be one of the main agents (Varga *et al.* 1988).

Auxins like 2,4-D and indole acetic acid (IAA) were found to increase the methylation of DNA (LoSchiavo *et al.* 1989, Arnholdt-Schmitt *et al.* 1991). In recent years, molecular markers are being used extensively to identify the DNA variations and for fingerprinting (Welsh and McClelland 1990, Brown *et al.* 1993, Ghareyazie *et al.* 1995). Use of RAPD primers for analysis of *Lathyrus sativus* somaclones showed that a single primer is not useful to generate unique DNA fingerprint, where a stepwise use of specific primers helped in fingerprinting the lines (Mandal *et al.* 1996). Analysis of beet somaclones (Munthali *et al.* 1996, Sabir *et al.* 1992) and tomato somaclones (Rus-Kortekaas *et al.* 1994) have employed RAPD and microsatellites to test for their usefulness.

The auxin 2,4-D is the most widely used phytohormone in rice cell culture studies. In view of variations caused by this auxin, a study was initiated to assess the extent of DNA changes in the regenerants, derived in the callusing media having low (0.5 mg dm^{-3}) and high (2.0 mg dm^{-3}) concentrations of 2,4-D. The study was also extended to fingerprint some of the advance generation of semi-dwarf somaclones of cv. B-370.

Materials and methods

Plants: Seeds of the *basmati* rice (*Oryza sativa* L.) cv. B-370 were obtained from a stock of seeds harvested from a pure line plot and stored for about 6 months at room temperature prior to use. Selected seeds, were manually dehulled and surface sterilised in 70 % ethanol for 1 min and then with 0.1 % $HgCl_2$ for 10 min. After thorough washing with sterile distilled water for 4 - 5 times, the seeds were left overnight in sterile water and used for excising embryos the next day. Isolated embryos were plated on modified MS semi-solid medium (Raina *et al.* 1987) supplemented with two different combinations of phytohormones: *1*) 2,4-D (0.5 mg dm^{-3}), NAA (2.5 mg dm^{-3}) and kinetin (0.5 mg dm^{-3}); *2*) 2,4-D (2 mg dm^{-3}) and kinetin (0.5 mg dm^{-3}). After about four weeks of incubation at 25 ± 1 °C in darkness, the embryo derived calli were transferred to regeneration medium (Raina *et al.* 1987) for plant regeneration under 12 h photoperiod (30 - 45 μmol m^{-2} s^{-1}). The regenerants that had grown to about 10 cm height were transferred to 1/4 strength MS medium for rooting in 20 cm long (22 × 150 mm) glass culture tubes. The rooted plants were transferred to 1/10 MS liquid medium (without sucrose, vitamins and amino acids) for hardening. After 10 - 15 d, they were transferred to pots with soil and grown to maturity in the greenhouse. Seed derived plants were raised from the same stock of B-370 seeds used for *in vitro* study, and also from three other *basmati* cultivars: Karnal Local, Type-3 and B-385 seeds of which were obtained from pure line plots of the Rice Breeding group of this Institute. Individual plants were raised in soil and grown to maturity.

A total of 12 regenerants were picked randomly from more than 30 regenerants derived from the 2 phytohormone treatments and used for molecular analysis. Seed grown plants of B-370 and Pusa Basmati 1 were used as controls, and seed derived plants from other cultivars (Karnal Local, Type-3 and B-385) were included for determining the intercultivar differences. In addition, 15 semi-dwarf somaclonal variants selected from over 1000 regenerants of B-370 obtained from a previous study (Raina *et al.* 1987) were also included for analysis. These somaclonal lines were in sixth or eighth generation of selfing. Two plants were picked up randomly from each of these lines, grown in 10 line plots with each line having 20 plants.

DNA preparation: Fresh leaf tissues of the regenerants and that of the seed raised B-370 and Pusa Basmati 1 plants were used for DNA extraction following the protocol of Saghai-Maroof *et al.* (1984). DNA concentration was quantified by gel electrophoresis and measuring absorbance at 260 and 280 nm using spectrophotometer (*Beckman Instrum. Inc.*, Fullerton, USA).

Restriction digests, electrophoresis and Southern analysis: DNA samples (10 µg) from each of the plants were digested with 5 different restriction enzymes, *EcoR*I, *Xba*I, *Hind*III, *Msp*I and *Hpa*II (*Promega Corporation*, Madison, USA). The restriction digested samples were electrophoresed in 0.8 % agarose gels [1 V cm^{-1}, 16 h in 1X TAE buffer (Tris acetate 0.04 M, EDTA 0.001 M, pH 7.8)]. The gels were southern blotted onto positively charged nylon membrane (*Hybond N+*, *Amersham Int.*, Buckinghamshire, UK) as per manufacturer's recommended procedures. Filters were prehybridized at 65 °C for 8 h and hybridised with the nick translated probe DNA (rice genomic clones RG-139, -207, -214, -303, -345 and -386) for 12 h. After hybridisation the filters were washed using 2X SSC (0.3 M NaCl, 0.055 M sodium citrate, pH 7.0) containing 0.1 % SDS (sodium dodecyl sulphate) at room temperature for 5 min followed by a wash at 65 °C using 1X SSC (0.15 M NaCl, 0.027 M sodium citrate, pH 7.0), containing 0.1 % SDS (sodium dodecyl sulphate) for 2 min and with 0.5X SSC (0.075 M NaCl, 0.014 M sodium citrate, pH 7.0) for 2 min. Autoradiographs were made at -70 °C using intensifying screens.

Random amplified polymorphic DNA analysis: Polymerase chain reactions were carried out in a 0.025 cm^3 reaction volume containing 40 ng DNA, 50 µM of each dNTPs (*Perkin-Elmer Corp.*, Norwalk, USA), 30 ng of ten-mer primer (*OPERON Tech*, Alameda, USA), 1.5 U Taq DNA polymerase (*Promega Corporation*, Madison, WI, USA), 1X Taq polymerase reaction buffer containing 50 mM KCl, 10 mM Tris-HCl at pH 9, 1 % Triton X 100 and 1.5 mM $MgCl_2$. The reaction mixes were aliquoted into 0.2 cm^3 tubes (*Micro-Amp* reaction tubes, *Perkin-Elmer Corp.*, Norwalk, USA) and amplification reaction was performed in a thermalcycler (*Gene Amp PCR system 9600*, *Perkin-Elmer Corp.*, Norwalk, USA) programmed for 40 cycles of 1 min at 92 °C, 1 min at 37 °C and 2 min at 72 °C for denaturation, primer annealing, and extension, respectively. The final extension reaction at the 40^{th} cycle at 72 °C was carried out for a total of 5 min. The amplification products were separated in 1.2 % agarose gels (*Sigma Biosciences*, St. Louis, USA) in 1X TAE

buffer, detected by ethidium bromide staining and recorded on *Polaroid* films (*Polaroid type 667* film, *Polaroid Corp.*, Cambridge, MA, USA). For a few of the primers (OPA18, OPD15, OPD20 and OPG8) the RAPD amplification products were digested with restriction enzyme *Taq*I (*Stratagene*, La Jolla, CA, USA) and electrophoresed in 8 % nondenaturing polyacrylamide gel at 90 V for 3 h.

Results and discussion

Effect of high 2,4-D supplemented medium on DNA variation in somaclones: For RFLP analysis, 12 regenerants of rice cv. B-370, grown in 0.5 and 2 mg dm^{-3} 2,4-D containing media, were tested with 7 probe-enzyme combinations. In comparison to the DNA samples from control plants, the *in vitro* derived regenerants revealed molecular changes. Polymorphic DNA bands were detected more distinctly with probe/enzyme combinations *RG207/EcoRI* and *RG303/EcoRI* among plants derived from high 2,4-D medium (Fig. 1*A*, *B*) whereas RG139/*EcoR*I, RG303/*Hind*III and RG386/*Hpa*II detected more polymorphic bands among low 2,4-D derived plants. The probe/enzyme combinations of RG214/*Xba*I and RG345/*Msp*I failed to reveal any polymorphism among the regenerants analysed but have shown polymorphism when compared with the control seed derived plants of cv. B-370 (Table 1). The number of hybridising bands detected in somaclones were more in comparison to that seen in the control plant DNA. Thus, *in vitro* culture has resulted in creation of more restriction enzyme sites in the DNA. This could be either due to addition or substitution of nucleotides thereby creating sites for restriction enzymes or due to insertion of DNA fragments (transposition) bearing the appropriate restriction enzyme sites. Tissue culture stress can result in either increased methylation of DNA which act as potential mutation hotspots or direct rearrangement of DNA sequences that are later inactivated transcriptionally so as to deter activation of potentially lethal genes (Muller *et al.* 1990). A direct role of transposons cannot be stated but a definite role of methylation of DNA sequences in the regenerated rice plants compared to control plants have been found (Muller *et al.* 1990).

Table 1. RFLP analysis of rice regenerants (cv. B-370) grown at high (2 mg dm^{-3}) and low (0.5 mg dm^{-3}) concentrations of 2,4-D in comparison with seedlings the same cultivar.

Probe	Enzyme	0.5 mg dm^{-3} 2,4-D size of bands [kb]	polymorphic band	2 mg dm^{-3} 2,4-D size of bands [kb]	polymorphic band	Control size of band [kb]
RG139	*EcoR*I	5.2,2,1.6	5.2 (3)*	5.2, 2, 1.6	5.2 (2)*	8.62
RG207	*EcoR*I	10,6, 2.5	6 , 2.5 (2)*	10, 6, 2.5	6, 2.5 (3)*	10
RG214	*Xba*I	0.6	-	0.6	-	6.2
RG303	*EcoR*I	9.7,8.6, 4.5	8.6,4.5 (2)*	9.7, 8.6, 4.5	8.6, 4.5 (4)*	9.7
RG303	*Hind*III	12.2,3, 2.3	12.2, 2.3 (4)*	12.2, 3, 2.3	12.2, 2.3 (2)*	3
RG345	*Msp*I	1.5, 1.0	-	1.5, 1.0	-	1.5
RG386	*Hpa*II	8.2, 1.8	8.2 (4)*	1.8	-	1.8

* Number of plants showing polymorphic bands within the parentheses.

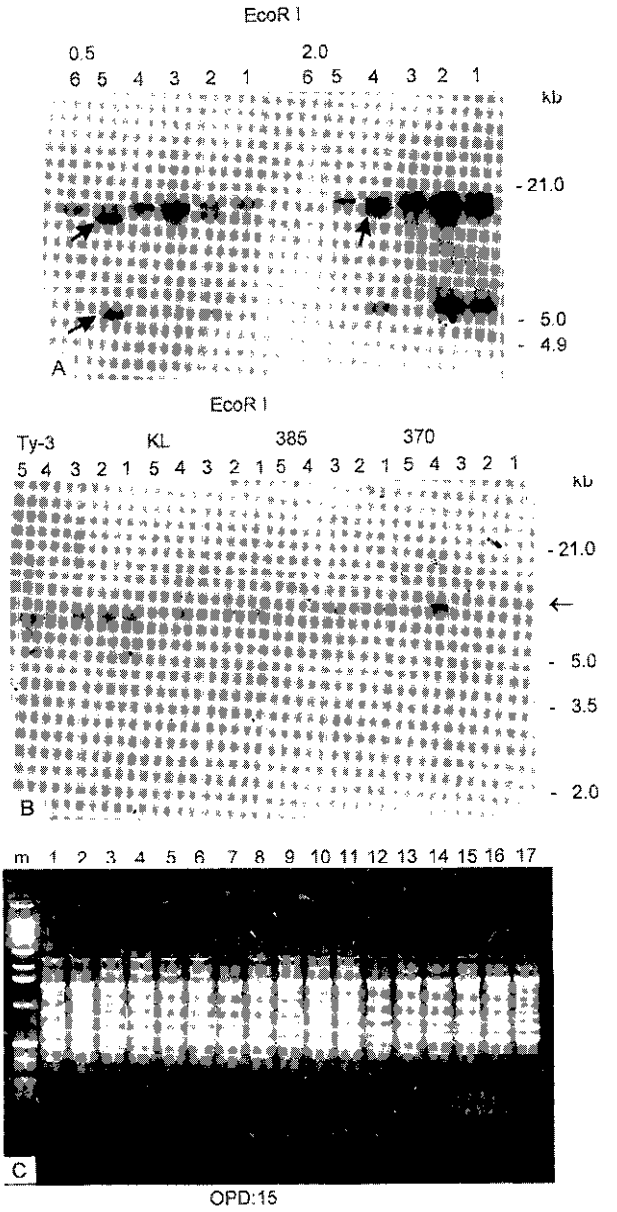


Fig. 1*A*. DNA isolated from the rice regenerants (cv. B-370) grown at 0.5 and 2 mg dm^{-3} 2,4-D was digested with *EcoR*I and probed with RG303. *Arrow* indicates the polymorphic bands. *B* - DNA isolated from seedlings of cvs. Karnal local, Type 3, B-385 and B-370 were digested with *EcoR*I and probed with RG303. Five individual plants of each cultivar was used for analysis. *C* - RAPD amplification profile obtained with primer OPD15 using DNA samples of regenerants (cv. B-370) grown at 0.5 mg dm^{-3} (lanes 1 - 6) and 2 mg dm^{-3} (lanes 7 - 12) 2,4-D and control plants (lanes 13 - 17). Lane m - 1 kb ladder.

However, in this analysis, the effect of high 2,4-D in the medium in inducing these molecular variations could not be conclusively derived since the results showed equal variations among the somaclones derived from 0.5 and 2 mg dm^{-3} 2,4-D containing media.

By RAPD analysis, majority of the amplification profiles did not reveal any differences between the somaclones raised on two media (Fig. 1*C*). When tested for polymorphism among the 88 amplified DNA bands obtained from amplification by 15 RAPD primers, only 9 polymorphic DNA bands could be detected out of a total of 1056 bands scored (number of regenerants × number of primers analysed). Amplification profiles of four primers revealed differences between the control plants and somaclones. Primer OPG7 produced a maximum of ten amplified DNA bands (strong and weak) of which three bands were polymorphic among the *in vitro* derived plants. The plants derived from 0.5 mg dm^{-3} 2,4-D showed band absence whereas the plants of 2 mg dm^{-3} 2,4-D medium showed presence or absence of the polymorphic bands. Minor polymorphic variations were also detected with the primer's OPG12 and OPG14 among the derived somaclones (Table 2). Using OPG16, variation could be detected between the somaclones and the control plants. In comparison to RFLP

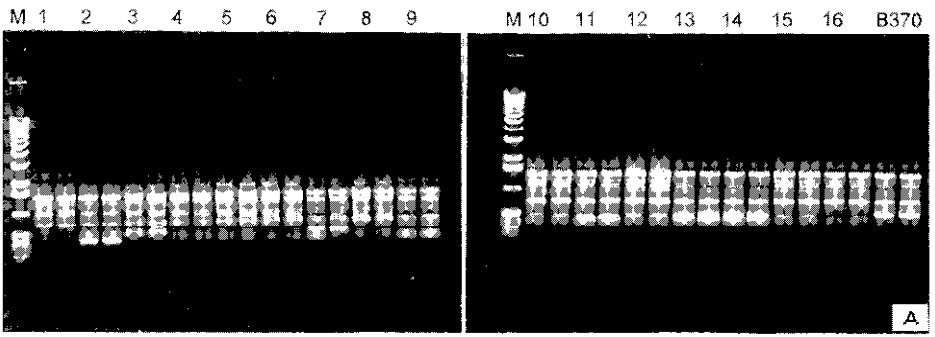
Table 2. RAPD analysis of rice regenerants (cv. B-370) grown at high (2 mg dm^{-3}) and low (0.5 mg dm^{-3}) concentrations of 2,4-D in comparison with control seedlings.

Primer	Sequence 5' to 3'	Number of amplified DNA bands	Number of polymorphic DNA bands
OPD15	CATCCGTGCT	9	-
OPD18	GAGAGCCAAC	6	-
OPD20	ACCCGGTCAC	6	-
OPE14	TGCGGCTGAG	4	-
OPE16	GGTGACTGTG	5	-
OPF02	GAGGATCCCT	Not amplified	-
OPF04	GGTGATCAGG	7	2
OPF16	GGAGTACTGG	5	-
OPF19	CCTCTAGACC	3	-
OPG06	GTGCCTAACC	5	-
OPG07	GAACCTAACC	10	3
OPG08	TCACGTCCAC	3	-
OPG09	CTGACGTCAC	6	-
OPG12	CAGCTCACGA	7	1
OPG14	GGATGAGACC	7	2
OPG16	AGCGTCCTCC	5	1
		88	9

analysis the RAPD analysis was thus found to be better since a larger number of amplified DNA bands can be examined within a short interval. A greater percentage of variation at 10.2 % was observed among the somaclones in comparison to the reports by Sabir *et al.* (1992) and Munthali *et al.* (1996) on beet somaclones.

Although, the percentage variation detected is high, the role of high 2,4-D in the media in inducing these variations could not be pinpointed.

In vitro culture thus increased the number of sites in the genome where primers can anneal to initiate amplification of DNA as compared to fewer sites in the control plant DNA. Our results agree with the reports of Rus-Kortekaas *et al.* (1994) where they have found RAPDs to be unsuitable for analysis of tomato somaclones.



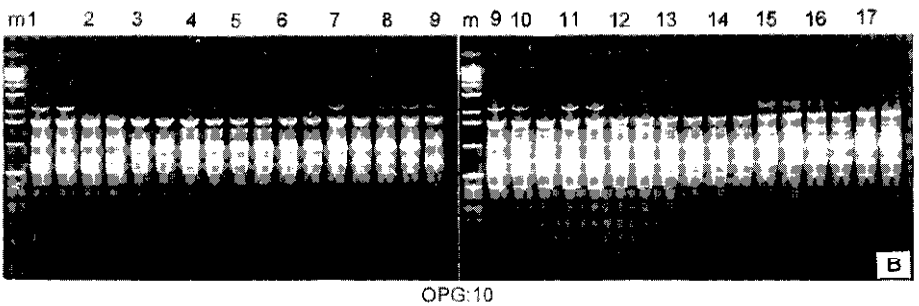


Fig. 2*A*. RAPD amplification profile obtained with primer OPD15 using DNA samples of somaclones of cv. B-370. Two individual plants of each of the fifteen somaclones (lanes 1,2,4-16; listed in Table 3*B*) were analysed. Lane 3 - cv. Pusa Basmati 1, lane 17 - cv. B-370 and lane M - 1 kb ladder. *B* - RAPD amplification profile obtained with primer OPG10 using DNA samples of somaclones of cv. B-370. Two individual plants of each of the 15 somaclones (lane 1, 2, 4-16; listed in Table 3*B*) were analysed. Lane 3 - cv. Pusa Basmati 1, lane 17 - cv. B-370 and lane m - 1 kb ladder.

In PCR based RFLP analysis, the three random primers (OPD15, OPD20 and OPG8) chosen were those whose amplification profile did not reveal any polymorphism. However, when tested for the efficiency of PCR based RFLP analysis, an increased number of DNA bands was observed. Amplification products of primer OPD15 produced a total of 21 DNA bands of which 3.5 kb and 2.5 kb bands in somaclones were found to be polymorphic (data not shown). A total of

17 bands were produced with OPG8 of which 5 were found to be polymorphic (3, 2, 1.4, 1.3 and 0.3 kb) in somaclones compared to only three monomorphic bands in the undigested product. Although OPD20 produced a total of ten DNA bands but none of these were polymorphic. Using the PCR based RFLP approach the three primers thus revealed a total of 48 DNA bands, of which 7 were found to be polymorphic, increasing thereby the detection limit of polymorphism to 14 %.

PCR amplification of DNA using either specific gene primer or arbitrary primers often do not reveal any polymorphic variation among closely related genotypes analysed. However, when the amplified products were digested with restriction enzymes (*Taq*I, *Hin*fI, *Rsa*I) that recognise either four or five base sequences and electrophoresed in polyacrylamide gels, molecular variations could be detected. Obviously, among the PCR amplified products, minor nucleotide substitutions which create or destroy a restriction enzyme recognition site is not unravelled in undigested samples (Ghareyazie *et al.* 1995).

This preliminary analysis has therefore revealed a larger number of DNA bands than RAPD analysis alone. Use of gene specific primers instead of random primers may help to locate the variant alleles in the regenerants with better efficiency. Consequently, it may be possible to detect, with better precision, the differences if any between regenerants derived from a high 2,4-D supplemented callusing medium and those derived from a low 2,4-D medium.

The above three molecular markers although helped to reveal polymorphic DNA bands in the somaclones compared to untreated plants, the effect of higher auxin induced molecular variations could not be conclusively derived. Also none of these techniques revealed any differences among the *basmati* cultivars analysed.

Molecular markers to fingerprint the rice somaclones: Amplification using 20 primers produced a total of 167 scorable bands out of which 96 bands were found to be polymorphic. The overall frequency of detection of polymorphism was found to be 57.48 % (Table 3*A*). A total of 11 primers revealed nonuniformity of the amplification profile among the lines analysed. Among the 15 somaclonal lines analysed nonuniform amplification between the individual plants of each line was detected in 9 lines using primers OPD16 and OPA17, in 6 lines using primer OPJ6, in 5 lines using primer OPG11, in 4 lines using primers OPG5 and OPD18, in 2 lines using primers OPG6 and OPG7 and lastly in only 1 somaclonal line using primers OPJ5 and OPD15. Uniformity of DNA amplification profile among the individual plants of each line was found using 6 random primers (OPA16, OPA18, OPD20, OPG2, OPG8 and OPG10). Among the primers analysed 13 primers (OPA16, OPA18, OPD15, OPD18, OPD19, OPD20, OPG2, OPG3, OPG6, OPG8, OPG10, OPJ5 and OPJ6) produced strong amplified DNA bands when used in RAPD analysis (Fig 2*A*, *B*). However only 6 of these detected uniformity in amplification profile between the plants. We expected fewer heterozygosities between the individual plants of a somaclone since the DNA samples were collected from the plants in their R8 generation. However, nonuniformity was detected in the amplification profiles of 2 randomly selected plants from each line. The somaclones which were conspicuous by showing nonuniform amplification profile are E170A, 513/E349A, 20/E42D,

Table 3*A*. RAPD analysis of rice somaclones.

Primer	Sequence 5' to 3'	Number of bands scored	Number of polymorphic bands scored
OPA16	AGCCAGCGAA	8	6
OPA17	GACCGCTTGT	11	6
OPA18	AGGTGACCGT	12	6
OPD14	CTTCCCCAAG	Not amplified	0
OPD15	CATCCGTGCT	9	4
OPD16	AGGGCGTAAG	6	5
OPD17	TTTCCCACGG	Not amplified	0
OPD18	GAGAGCCAAC	9	5
OPD19	CTGGGGACTT	11	6
OPD20	ACCCGGTCAC	9	6
OPG02	GGCACTGAGG	10	5
OPG03	GAGCCCTCCA	6	2
OPG05	CTGAGACGGA	8	2
OPG06	GTGCCTAACC	4	3
OPG07	GAACCTGCGG	17	12
OPG08	TCACGTCCAC	6	3
OPG10	AGGGCCGTCT	11	6
OPG11	TGCCCGTCGT	8	6
OPJ05	CTCCATGGGG	8	3
OPJ06	TCGTTCCGCA	7	3
		167	96

Table 3*B*. Analysed results of RAPD generated bands in rice somaclones, cvs. B-370 and Pusa B 1.

Description of plants	Bands scored	Average number of bands	Polymorphic bands	Unique bands	Number of markers	Primers identifying heterogeneity
49/E43 A	114	6.3	8	2	1	1
E 170A	125	6.9	30	10	4	3
Pusa B 1	120	6.6	37	10	1	1
24/E54B	117	6.5	32	3	1	1
513/E349A	118	6.5	33	8	1	4
20/E42T	119	6.6	30	8	1	4
20/E42D	119	6.6	29	7	1	6
493/E241	114	6.3	29	9	4	2
493/E305A	112	6.2	34	7	5	4
509/E346A	103	5.7	18	2	1	4
423/E302A	119	6.6	29	10	1	3
92/E174A	112	5.7	21	5	1	4
543/E246	95	5.2	30	4	1	3
544/E247A	91	5.05	28	5	0	2
482/E236	102	5.6	17	6	1	5
76/E166A	107	5.9	19	4	1	4
B 370	103	5.7	21	9	3	0

20/E42T, 493/E305A, 509/E346A, 92/E174A, 482/E236 and 76/E166A. Only 8 primers were found to be suitable for preliminary fingerprinting analysis. The RAPD data when analysed on an individual plant basis, the number of amplified DNA fragments ranged from 91 to 125. The average number of detected fragments per primer was in the range of 5.05 to 6.9. The number of polymorphic DNA bands ranged from a low of 8 (49/E43A) to a maximum of 37 in Pusa Basmati 1. The present study detected a total of 32 unique DNA bands. The number of unique amplified DNA bands in individual somaclone which can be used for fingerprinting was found to range from 1 to 5, except in somaclone 544/E247A for which no unique band could be found (Table 3*B*). Although we observed 80 - 90 % similarity in the amplification profile of these somaclones using RAPD analysis, a clear grouping was evident between the lines that resemble R-370 (E170A, 20/E42D, 49/E305A, 509/E346A, 423/E305A, 543/E246 and 544/E247A) and those which differ [similar result was obtained with RFLP analysis using RG303/*Xba*I; RG303/*Eco*RV combination and PCR based RFLP analysis using OPA18/*Taq*I combination (data not shown)]. The somaclones 49/E43A, 24/E54B, 513/E349A, 20/E42T, 493/E241, 92/E175A, 482/E236 and 76/E166A were found to resemble the amplification profile of Pusa Basmati 1. This grouping may be a chance event during *in vitro* culture where the DNA variation is random or could be due to selection of *basmati* traits in the somaclones when they were advanced in each year as the most superior compared to their counterparts having inferior qualities. In this analysis we were successful in analysing the lines using RAPD primers and amplification profile was repeatable. However, fingerprinting of the somaclones could not be accomplished since none of the primers produced amplification profiles that are unique for each of the lines. Thus the RAPD primers only helped in finding the similarity and differences among the somaclones.

The study shows that *in vitro* induced molecular changes in rice somaclones can be detected using a PCR based technique. The PCR approach permits a more accurate estimation of the rate of nucleotide substitution that seems to be of common occurrence during a tissue culture phase. However, RAPD analysis alone is not found useful to detect molecular variation in the somaclones. In this situation a combination of various marker techniques can help as revealed in PCR based RFLP analysis.

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