Induction of somaclonal variation by tissue culture and cytogenetic analysis in *Oryza sativa* L.

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Abstract

Protocols were developed for plant regeneration from callus induced in mature embryos of rice. Somaclonal variation was scored by genome mutation, chromosome mutation and plasmon mutation in R_0, R_1 and R_2 plant progenies. The frequency of haploids and diploids appeared in the ratio of 20:33. Variation in the chromosome number in callus cells was found to be high and age dependent. Different types of chlorophyll deficient mutants including albinos appeared in R_2 plant progeny where gene mutation frequency was the highest (52.4 %). The results revealed that a high frequency of somaclonal variation is possible to generate by tissue culture techniques.

Additional key words: chlorophyll deficient mutant, chromosome mutation, gene mutation, plasmon mutation, rice.

Introduction

A break through in rice tissue culture occurred in 1968 when plants were regenerated from calli obtained from mature and immature embryos, roots and seeds, and when haploid plants were regenerated from anther culture. Triploid rice plants were also produced soon after by endosperm culture. Variation was often observed among the regenerated plants and their progenies (Oono 1975, Chen and Li 1978, Abarbanell and Breiman 1989, Coppens and Devitte 1990, Pareddy and Petolino 1990, Redway *et al.* 1990, Sandhu *et al.* 1994, Abbasi *et al.* 1995). Extensive efforts were made to improve anther and somatic cell culture efficiency. This was established by the development of the efficient culture media like the MS medium, Gamborg's medium and the N_6 medium (Chu *et al.* 1975) for rice. It has also been

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Abbreviations: 2,4-D - 2,4-dichlorophenoxyacetic acid, NAA - naphtalene-1-acetic acid, BAP - 6-benzylaminopurine, CW - coconut water, CH - casein hydrolysate, Kin - kinetin, MS - Murashige and Skoog (1962).

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established that gameto- and somaclonal variabilities can be efficiently generated by *in vitro* cultures. Somaclonal variations can be determined in various ways like mutations, heritable changes in DNA sequence (base substitution, insertion, deletion or rearrangement) or in DNA quantity and conveniently classified as genome, chromosome, gene and plasmon mutations.

The occurrence of mutation during the tissue culture has been shown by analysis of single cell derived clones and regenerated plants and their progenies. Several organ modifications have been recorded which are either dominant or recessive gene controlled traits.

As regards genome mutation, variation in ploidy levels occur in plants regenerated from anther and embryogenic calli cultures. Observations made on callus cells revealed that chromosome number in every callus cell is remarkably different regardless of the growth regulators used. Some cells showed more than 50 chromosomes instead of 2n = 24 in rice. Polyploid and aneuploid cells frequently appear (Bajaj and Bidani 1980, Ying 1986). Chromosome mutation is a common phenomenon in tissue culture of many plant species including rice. Multiple and reciprocal translocations involving several non-homologous chromosomes from somatic cell cultures of rice cultivars have been reported.

Gene mutation has been well studied in rice calli induced from seeds. The pattern of appearance of mutants further differ and assort in the R_2 and R_3 generations.

As regards plasmon mutation, albino plants are considered which frequently regenerate from tissue culture. Albinos result from mutation in chloroplast DNA. Electrophoresis of chlorophyll protein complexes produced several bands in green plants but few in albino plants (Oono 1975, Nitzsche and Wenzel 1977, Oono *et al.* 1984, Day and Ellis 1984, Dutta *et al.* 1990).

Changes in the nuclear genome during differentiation are analysed to investigate the cause of somatic mutations. Rice nuclear DNA of developing embryos and 30-d-old callus digested with restriction endonuclease exhibited different restriction patterns. Callus DNA showed many discrete bands suggesting that some of the DNA sequences had been amplified during callus induction (Hanzel *et al.* 1985, Thompson *et al.* 1986).

Numerous studies have been undertaken modifying the composition of the culture medium, the culture environment and the selection of inoculum *in vitro* culture. The aim of this investigation was standardization of an *in vitro* rice regeneration and screening of occurrence of somaclonal variation.

Materials and methods

Seeds of *Oryza sativa* L. cv. IR 72 were dehusked and washed by running tap water for 90 min. To avoid contamination the seeds were dipped in extran for 5 min, washed in tap water and surface sterilised with 70 % alcohol for 30 s followed by 0.1 % mercuric chloride + Tween 80 (0.1 %) for 15 min or in sterile distilled water. Washing of explants before planting with an antioxidant solution containing 150 mg dm^{-3} citric acid (pH 2.5) and 100 mg dm^{-3} ascorbic acid (pH 2.9) for

30 - 60 min was found necessary to prevent browning of callus. Embryos ranging in size 0.2 - 0.3 cm aseptically cut out from the seeds were inserted on the MS medium (pH 5.8) solidified with *Difco-Bacto* agar (7 g dm^{-3}). MS medium was supplemented with different concentrations of growth regulators (2.5 mg dm^{-3} 2,4-D, 0.5 - 3.0 mg dm^{-3} NAA, 1 - 2 mg dm^{-3} BAP, 1 - 2 mg dm^{-3} Kin), 15 - 20 % CW, 500 mg dm^{-3} CH and 30 g dm^{-3} saccharose. The media were dispensed in culture tubes plugged with cotton and autoclaved at 121 °C for 15 min. Four embryos were inoculated in each culture tube (20 × 150 mm) containing 15 cm^3 of medium. Cultures were incubated at 26 ± 1 °C, 16/8 h light/dark cycle (irradiance of 42.25 µmol m^{-2} s^{-1}) and ambient air relative humidity of 55 %. Each treatment consisted of at least 12 replicates and was repeated more than three times. Callus was usually induced within one month after incubation. Subcultures at intervals of 1 - 3 weeks were carried out to reduce browning. Inoculated callus weighed approximately 150 - 200 mg.

For studying organogenesis callus was subcultured on a differentiation medium (MS − 0.5 mg dm^{-3} 2,4-D + 15 % CW + 500 mg dm^{-3} CH) and maintained for 7 - 8 weeks. Then callus was transferred to MS − Kin when shoot buds emerged. The young plantlets were then transferred to half concentration of growth regulators and finally to hormone free MS medium to establish a vigorous root system. Regenerated plants were transferred to vermiculite, soil and peat mixture and grown till seed maturity.

For root formation and cytological study young plantlets were transferred to half strength MS medium supplemented with NAA (0.5, 1.5, 2 and 3 mg dm^{-3}). Root system including primary and secondary roots was observed upto a length of 0.2 - 0.5 cm. For cytological study root tips were pretreated with a saturated solution of α-bromo-naphtalene, fixed in acetic ethanol and squashed in acetocarmine. Chromosome number and aberrations were regularly scored from the temporary slide preparations. After plant regeneration R_0, R_1 and R_2 plants were observed for scoring mutation representing somaclonal variation.

For the evaluation of statistical significance of differences Student's *t*-test was applied. Mutants like fragrant flower (frg), scented endosperm (sk), waxy endosperm (wx), translucent endosperm (wb), mutation in physiological characters like earlyness in growth (Ef), lateness (Lf), mutant traits like clustered spikelets (CL), dwarfs (d) and compact panicle (Dp) appearing in R_2 regenerants were scored by visual observation.

Results

Standardization of tissue culture was evidenced by seed germination, callus induction, root formation, shoot multiplication and plant regeneration (Table 1, 2) in R_0 and R_1 cultures. Embryo germination was recorded to be as high as 90 %. Callusing efficiency of mature embryos was observed on the MS medium supplemented with 2,4-D. Callus induction was in the range of 8.52 - 17.00 % with a total of 59.25 % over observations (Table 1).

Recovery of plantlets was in the range of 6.31 - 29.97 %. Complete plantlet recovery was affected due to drying during differentiation (Table 2). Different kinds

of mutation, *i.e.*, genome mutation, gene mutation and plasmon mutation were observed in callus as well as in regenerated plants.

Table 1. Callus induction [%] on mature embryos of rice on MS medium with common supplements 2.5 mg dm^{-3} 2,4-D, 500 mg dm^{-3} CH, and 30 mg dm^{-3} saccharose and different concentrations of NAA [mg dm^{-3}], BAP [mg dm^{-3}], Kin [mg dm^{-3}] and CW [%].

NAA	BAP	Kin	CW	Callus induction [%]	Nature of callus
0.50	1.00	1.00	15	15.50	soft, friable, compact, nodular
1.50	1.25	1.25	17	17.00	
2.00	1.50	1.50	18	12.40	soft friable
2.50	1.75	1.75	19	10.00	soft friable / crystalline
3.00	2.00	2.00	20	8.52	crystalline

Table 2. Organogenesis and parameters of plantlet growth under different concentrations [mg dm^{-3}] of NAA, BAP and Kin in differentiation and transfer media of MS with common supplements of 0.5 mg dm^{-3}, 2,4-D, 500 mg dm^{-3} CH, and 30 mg dm^{-3} saccharose. For root formation half-strength MS + NAA; for shoot formation MS + BAP + Kin.

NAA	BAP	Kin	Root number [plantlet^{-1}]	Plantlet number	Root length [cm]	Shoot number [plantlet^{-1}]	Shoot length [cm]	Complete plantlet number
0.50	1.00	1.00	3 - 5	70	0.2 - 0.5	45	5.08-10.20	20
1.50	1.25	1.50	1 - 2	87	0.2 - 1.0	96	2.54-12.70	39
2.00	1.50	1.75	2 - 3	247	0.5 - 1.5	20	2.54- 5.08	4
2.50	2.00	2.00	2	190	0.5 - 1.0	70	1.27- 5.08	16
3.00	2.25	2.25	1 - 3	40	0.2 - 0.5	-		-

As regards genome mutation frequency of both haploids and diploids were significantly high in the R_1 plants (Table 3). However haploids were significantly reduced in height and panicle length as compared to the diploids; the frequency of haploids and diploids being in the ratio of 20:33.

As regards chromosome mutation in callus of different ages, chromosome number was found to be increased from 2n = 24 in normal to 2n = 46 to 48 (7- and 10-month-old calli produced tetraploids). However, age of callus was not found to be a factor for increased chromosome number because the normal number was also found in the older age groups. The leaf colour of tetraploids appeared darker than that of normal ones (Table 4).

As regards genome mutation, chromosome number in callus cells were counted and variation was found to be quite high ranging from 12 to 32; and among 750 cells counted, 100 cells showed normal diploid number while the remaining 650 cells showed varied number of chromosomes among which largest number of cells showed the haploid number of chromosomes (Table 5).

As regards plasmon mutation several types of variation were observed among the R_2 plant progeny, particularly different types of chlorophyll deficient mutation

(Table 6). Total number of mutations was 9.76 % (44 mutant plants out of 450 R_2 panicles examined) among which appearance of albino seedlings were highest followed by xantha, viridis, chlorina and striata.

Table 3. Frequency of haploids and diploids and their growth parameters (means ± SE) in the vegetatively propagated R_1 plants under different height classes.

Number of plants		Haploid		Diploid	
n	2n	height [cm]	panicle length [cm]	height [cm]	panicle length [cm]
7	6	50.4*	10.4 ± 6.05	64.3 ± 2.00	12.9 ± 1.80
5	10	51.4 ± 4.32	9.0 ± 2.01	78.0 ± 3.30	14.0 ± 1.95
3	8	52.4 ± 4.00	10.7 ± 5.00	83.7 ± 2.01	15.0 ± 1.00
2	4	64.3*	11.3 ± 6.00	100.6 ± 2.53	21.8 ± 2.06
3	5	69.7*	13.7 ± 4.78	104.5*	22.2 ± 2.00

* - due to lesser number of plants variation was not measured; $t_{[8]} = 3.5664$ and $15.7607 > P_{5\,\%}$ for n × 2n.

Table 4. Variation in chromosome number in callus of different age (mean of 500 cells; 5 replicates) of each age class).

Age of callus [month]	Chromosome [number]	Primary leaf colour	Age of callus [month]	Chromosome [number]	Primary leaf colour
7	46 ± 2.35	dark	10	48 ± 4.70	normal
7	48 ± 4.20	dark	10	24 ± 0.85	normal
7	46 ± 1.32	dark	10	24 ± 0.92	normal
7	46 ± 1.21	dark	10	24 ± 0.75	normal
7	46 ± 2.00	dark	10	24 ± 1.00	normal

$t_{[8]} = 4.7864 > P_{5\,\%}$ for $n_1 \times n_2$ in ages of callus

Table 5. Chromosome number variation (genome mutation) in callus cells (60 % of the cells showed haploid number of chromosomes; mean chromosome number = 19.20 ± 4.03).

Cells [number]	Chromosomes [number]	Cells [%]
50	16	6.66
100	24	13.33
150	32	20.00
200	12	26.66
250	12	33.33

Gene mutation was observed to be distributed as different types of mutants which were distinctly identified. Among the 500 R_1 panicles, 262 panicles produced R_2 plant mutants resulting 52.4 % mutation. Among the mutant types, compact panicle

mutant (Dp) showed the highest frequency followed by the dwarfs, lateness in growth and waxy endosperm, the lowest frequency being the mutants for earlyness in growth. A wide range of variation occurred due to the appearance of the wide range of gene mutation (Table 7).

Table 6. Chlorophyll mutation in R_2 plants from rice calli.

Panicles examined in R_1 [number]	Panicles mutated [number]	[%]	Types of mutation albino (al)	xantha (l-y)	viridis (v)	striata (ws)	chlorina (chl)
450	44	9.76	12	10	8	6	8

Table 7. Types of gene mutation in R_2 plants from rice calli. From 500 panicles examined, 262 panicles were mutated (52.4 %)

Type of mutations	[number]	[%]
Dwarfs (d)	57	11.4
Earlyness in flowering (Ef)	15	3.0
Lateness in flowering (Lf)	35	7.0
Fragrant flower (fgr)	5	1.0
Compact panicle (Dp)	60	12.0
Clustered spikelets (Cl)	17	3.4
Scented endosperms (sk)	23	4.6
Translucent endosperms (wb)	20	4.0
Waxy endosperm (wx)	30	6.0

Discussion

During the cell culture variations occured in several characters. Differences in callus induction among different cultivars were noticed in the earlier studies in rice tissue culture (Oono *et al.* 1984). Calli induced from rice seeds were classified into two groups, compact and friable. Variation in chromosome number was studied to ellucidate the origin of diploid plants and to investigate the source of the variation found in regenerated plants. Several ploidies like haploids, diploids, multiploids and mixoploids were observed. An increase in chromosome number during callus growth was also regularly observed which revealed that duplication of chromosome number is a common feature in rice tissue culture (Tables 4 and 5). Variation in chromosome number and also number of cells showing different numbers of chromosomes was observed in calli of different age groups. The haploid number of chromosomes occurs very often. The older calli had diploid number of chromosomes while younger calli had tetraploid number. This decreasing chromosome number in the later age group was due to processing and elimination of chromosome thereby showing organization of the stable genomic structure. In this investigation somatic mutation

and variation could be classified into 4 types: genome mutation, chromosome mutation, gene mutation and plasmon mutation.

The occurrence of the mutation during the tissue culture were detected through the analyses of single cell derived clones and regenerated plants and their progenies. It was observed that mutation and chromosome duplication occur during culture period of callus. Genome mutation was observed by variation in ploidy and chromosome number. Because of small size of rice chromosomes, chromosome mutation, a common phenomenon in tissue culture, could not be observed by multiple reciprocal translocations, although isolated regenerated plants with multiple reciprocal translocations involving 5 non-homologous chromosomes from somatic cell culture was reported earlier (Oono 1975).

The albino plants are classified as plasmon mutation. It is suspected that albinism results from mutation in chloroplast DNA. Plastid genome of albino plants regenerated from rice callus was analysed. It was observed in albino plants that their chloroplast DNA was totally absent and this was supported to be the major cause of albino regeneration in rice (Oono *et al.* 1984). Other mutations presented in Table 7 came under gene mutation obtained in R_2 plants from rice callus. The characters are often found to be controlled by major qualitative gene pairs and have been mapped in the rice linkage groups. 262 mutants were observed out of 500 panicles examined with a maximum frequency of 60 of the mutation, 'compact panicle' (Dp) while the mutant fragrant flower (fgr) had the lowest frequency. These were observed in the R_2 progeny and now under observation for the genetical analyses of the progenies of the reciprocal crosses made between the parents and progenies.

Mutants obtained by tissue culture have been found to be a good source of genetic variation for rice breeding. Regeneration of rice plants from embryonic callus tissue has become a routine work in plant breeding programmes.

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