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Rice improvement, involving altered flower structure more suitable to cross-pollination, using in vitro culture in combination with mutagenesis

S.-K. MIN

China National Rice Research Institute, Hangzhou, China

Abstract. Anther and somatic tissue culture in combination with mutagenesis were carried out to evaluate the efficiency of different mutagenic treatments of various in vitro culture materials, and to obtain some promising variants for rice improvement. Results indicated that in *japonica* rice radiation treatment of dry seeds and young panicles influenced the percentage of green plantlets regeneration from anther culture. Both treatments increased significantly the percentage of regenerated green plantlets in comparison with the control. Irradiation with 30 Gy of rice callus increased also the percentage of regenerated green plantlets. For *indica* rice, the combination of the suitable dose of gamma rays irradiation on seeds and an improved medium, increased the percentage of callus induction. This approach made it possible to use anther culture in *indica* rice breeding. Somatic tissue cultures combined with radiation-induced mutagenesis led to the development of a number of promising mutants including some new cytoplasm-nucleus interacting male-sterile lines with almost 100% stigma exsertion. Their development would be of practical significance for increasing the genetic diversity for production of hybrid rice.

Key words: anther culture, *Oryza sativa*, somatic cell culture.

Introduction

Since Ichijima's report on mutations induced by X-radiation in rice (ICHIJIMA 1934), many rice breeders have been interested in mutation research. In China, the first mutation variety was Ai-Fu 9 bred in 1964. Up to now, 85 rice varieties have been developed through induced mutations. Seven of

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Correspondence: S.-K. MIN, China National Rice Research Institute, Hangzhou, China.

them have reached over 100,000 ha annual extension area including two varieties, Yuanfengzao and Zhefu 802, with the largest extension area above 1.0 and 1.3 million ha, respectively.

Anther culture was started in China in 1970, and already more than 15 rice varieties have been developed by haploid techniques, among them *japonica* type Zhe-Keng 66 (6,700 hectares in 1985), Shong-Hua 8 and 9 (10,000 hectares in 1985). Up to now, the ratio of pollen-derived green plants to anther number was 0.5% for *indica* rice and 5-6% for *japonica* rice. Thus, breeding through anther culture has been relatively easy in *japonica* rice, but there are still hindrances to using this approach in *indica* rice. In order to increase the culture ability of *indica* rice, researches at the China National Rice Research Institute (CNRRI) are screening genotypes, improving inoculation methods, changing culture medium components and culture conditions such as light and pretreatment temperature of donor plants.

The potential usefulness of somaclonal variation for plant improvement became first apparent in sugarcane. In the Hawaiian Sugar Planters' Association Experimental Station the variability among plants derived from sugarcane tissue cultures was observed already in early 60s. Similarly, OONO reported in 1978 that the variation frequency of the plants regenerated from rice in vitro may reach 71.9%.

In 1979 the CNRRI started research on somaclonal variation by using young rice panicles and mature embryos. Somaclonal variation has many advantages, such as a relatively high frequency, wide range, and true breeding in the first self-pollinated progeny (SUN et al. 1983). Rice T42, a somaclonal line developed by CNRRI, covered an area of 1,500 hectares in 1985. In addition to somaclonal variations occurring in cell and tissue culture, a certain tendency for oriented selection of mutated cells is also included in the culture process. These research activities have day by day manifested their potential application for varietal improvement.

Rice is a strictly self-pollinating crop. The three lines used in the hybrid rice system at present, still keep the flower structure proper for selfing. The seed-setting percentage of outcrossing of existing male sterile lines with stigma exsertion 35% in the field is generally 20-30% for *indica* type and 40-50% for *japonica* type. It is estimated that when the seed-setting percentage is increased by 1%, the yield of produced hybrid seeds will be increased by 44-94 kg per hectare. So, there is a great potential to increase the seed pro-

duction of hybrid rice by improving the floral traits of three lines to make them more suitable to cross-pollination.

In general, a male sterile line with big and exserted stigmas, long duration of anthesis, as well as the maintainer line and restorer line with large anthers, large quantity of pollen and good anther dehiscence are needed. Quite a number of scientists have reported that the anther size, amount of pollen, stigma size and rate of stigma exsertion of some wild rice species are obviously more suitable for hybrid production than those of cultivated rice (SAMPATH 1962, OKA, MARISHIMA 1967, VIRMANI, ATHWAL 1973, PARIMAR et al. 1979, LI et al. 1981a). Within the cultivated rice, there also exists a somewhat wider variation of flower structure (XU et al. 1986). All of these provide rice germplasm resources for improving the floral structure.

A number of new lines which have floral structure suitable for outcrossing have been developed in China through hybridization between wild rice and cultivated varieties (LIN et al. 1981b). With this method the process is slow and new lines often have undesirable, linkaged characters and thus cannot meet practical needs. Therefore, it is necessary to adopt an integrated breeding technology for further development of the new lines with improved floral structure suitable for outcrossing.

The introduction of hybrid rice in China has brought a high economic efficiency, but almost 95% of the hybrid rice currently produced is from WA (wild abortive)-type sterile lines. This unitary of cyto-sterility can generate genetic vulnerability. Therefore, to develop non-WA-type, a search for a new germplasm of male-sterile lines has become a subject of research. By using in vitro culture, new male-sterile lines have been obtained, but they are similar to the WA-type (LING et al. 1988).

It has been proved that wide variation appears in populations obtained after mutagenic treatment and in vitro culture, and that anther culture can shorten the breeding cycle. Chinese scientists have conducted investigation on the effectiveness of combining in vitro culture and mutagenesis. It was found that through a combination of lower doses of radiation followed by anther culture, the frequency of callus induction and green plantlet regeneration can be increased (YIN et al. 1982). Besides, it was found that radiation treatment can increase variation in progenies coming from anther culture and somatic tissue culture (ZHAO et al. 1983).

Material and methods

Anther culture in combination with gamma-ray treatment

Four *japonica* rice (*Oryza sativa* L.) varieties, R856, R85221, Liu-Qian-Xin B and Xiu-Ling B were used as materials. Their seeds, young panicles and callus from anthers were irradiated with different ^{137}Cs gamma-rays doses. The doses 200, 250 and 300 Gy for seeds, 2, 4, 8 and 16 Gy for young panicles, and 30, 60 and 90 Gy for callus were applied. Unirradiated plant material was used as a control.

For *indica* rice, three crosses: GST-2/8798 (big anther and stigma), V41 B/8798 and 7944 (big anther) × Zhenshan 97B, have been used. Anther culture was also carried out for material from irradiated seeds planted in the field. In addition, the seeds of 11 wide compatibility varieties (WCV) and 11 photo-(thermo)-sensitive genetic male sterile rice (most of them being *indica* rice) were irradiated with 300 Gy gamma-rays, and then their anthers were incubated. The regenerated plants (T_1) and subsequent generations were planted in the field, and selected for altered floral structure.

The gamma-ray treatment of somatic cell culture

The germinating embryos and young panicles of Basmati 370 selection, 87-156 and Hu18 *indica* rice were used as explants in somatic cell culture. The callus with green spots and callus with developing shoots obtained through tissue culture of Basmati 370 selection were irradiated with 20, 50, and 100 Gy of ^{137}Cs gamma-rays (MIN et al. 1991). The male-sterile plants developed in this study, were used in the test crosses and then backcrossed for screening restorer and maintainer lines. The heterosis of F_1 hybrid rice has been investigated.

Results and discussion

Anther culture in combination with mutagenesis

Effect of gamma-ray treatment on callus induction and green plantlets regeneration of *japonica* rice

Within the tested range of doses, there was little effect of radiation treatment of dry seeds and young panicles on the percentage of callus induction. The average percentage of callus induction was lower than 2.0% in all cases, but the percentage of regenerated green plantlets from treated seeds and young panicles increased significantly in comparison to the untreated check (Table 1).

Table 1. Effect of gamma-ray treatment of seeds and young panicles on callus induction and green plantlets regeneration from anther cultures of four *japonica* rice varieties

	Dose (Gy) – Seed				Dose (Gy) – Young panicle				
	0	200	250	300	0	2	4	8	16
ACI (%)	1.8	1.4	1.5	1.0	1.0	1.2	1.3	1.5	1.2
AGPR (%)	24.5	36.5	39.1	34.8	34.6	64.6	54.4	47.7	53.8

ACIP (Average callus induction) – No. of calli / No. of inoculated anthers
AGPR (Average green plantlet regeneration) – No. of calli with green shoots / No. of calli inoculated

After irradiation with doses 0, 30, 60 and 90 Gy, calli were consistently cultured on the original medium (without sub-culturing) and transferred, when they grew to about 2 mm in length, to the redifferentiation medium for re-

Table 2. Effect of continuous transfer of callus without sub-culturing on green plantlets regeneration in *japonica* rice anther culture irradiated with 0, 30, 60 and 90 Gy of gamma-rays (the mean value for all treatments)

No. of transfers of calli to redifferentiation medium	Green plantlets (%)	Callus with green spots (%)	Albino plantlets (%)
1	17.1	8.6	42.9
2	18.1	19.0	56.0
3	21.0	13.5	48.7
4	22.8	6.6	44.7
5	24.2	1.7	29.2
6	21.2	2.9	29.8
7	8.6	0.0	18.1

generation of green plantlets. It was found that in this method callus could regenerate much more green plantlets till the 6th transfer (Table 2), but after that the callus grew slowly because of gradual reduction of nutrients in the original medium. The percentage of callus with green spots was also higher till the 3rd transfer.

The treatment with 30 Gy gave the highest percentage of regenerated green plantlets. Meanwhile, the percentage of callus with green spots in this treatment increased, and percentage of root-like and brown callus decreased obviously (Table 3).

Table 3. Effect of gamma-irradiation of anther cultures on callus formation and green plantlets regeneration (%)

Dosage (Gy)	Normal callus	Callus with green spots	Root-like callus	Brown callus	Green plantlet regeneration
0	12.8	64.3	9.5	13.4	16.4
30	9.5	81.0	5.3	4.2	33.9
60	3.1	77.1	6.2	13.6	11.0
90	7.3	73.3	7.3	12.1	18.2

Effect of gamma-ray treatment on callus induction and green plantlets regeneration of *indica* rice

In the past few years progress has been made in the development of *indica* rice anther cultures and their use in mutation techniques. Seeds of F_6 generation derived from three *indica* rice crosses were irradiated with 60 and 120 Gy, respectively. Anthers from these treatments, as well as the check variety, were inoculated on two different media, Kasha's medium and M-8 medium (MARSOLAIS, KASHA 1985). Table 4 indicates that the callus induction percentage in treatment with 120 Gy gamma-rays was higher than in any other treatments, and the result on M-8 medium was better than that on Kasha's medium.

Table 4. Effect of donor plants (F_6) seed irradiation with gamma-rays on callus induction in *indica* rice

Medium	Dosage (Gy)		
	0	60	120
Kasha's	0.26	0.10	1.67
M-8	3.02	2.54	4.43

We have obtained a lot of regenerated green plantlets, but many of them were haploids. It was inspiring that from the progeny of the regenerated green diploid plants we have obtained several completely sterile or semi-sterile plants with big and exserted stigmas as in parent varieties.

Since we can obtain a comparatively higher percentage of callus induction from anthers of *indica* rice, it was possible to breed new rice germplasms

through anther culture in combination with mutagenesis. A new research project on anther culture has been initiated with 11 wide compatibility varieties (WCV) and 11 photo-(thermo)-sensitive genetic male-sterile lines. This plant material was irradiated with a relatively high dose (300 Gy) applied on dry seeds. Mutants from this treatment are expected to be used in "two-lines" hybrid rice breeding in China.

Somatic cell culture in combination with mutagenesis – the development of male sterile lines

The frequency of male sterile lines by in vitro radiation

We used the dehulled seeds of Basmati 370 selection as materials (MIN et al. 1991). At the time when large quantity of green spots or developing green shoots were differentiated sooner or later, radiation treatment was done, and then the callus with green spots or developing green shoots was immediately sliced into small pieces uniform in size and transferred to the differentiated medium for continued growth into seedlings.

Table 5. Effect of in vitro culture irradiation on variability of rice

Treatment	Albino (%)	Generation	Frequencies of elite lines		
			no. of plant lines surveyed	elite lines (no.)	(%)
Somatic cell culture	0.6	2	222	2	0.9
Conventional radiation	2	4	1000	1	0.1
In vitro radiation	3.6	4	399	13	3.3

The seedlings in test tubes were transplanted into soil and more than two thousand regenerated plants (T1) were obtained. Two individual plants with complete sterility and almost complete stigma exsertion were discovered in the field among the 100 Gy group, and afterwards named as TB-A lines. Using the same method, we have also obtained 9 and 4 individual male-sterile plants from 87-156 and T18-6 breeding lines, respectively.

The transfer of TB-A male-sterile line

The transfer of TB-A male sterility was done in order to develop desirable completed male-sterile lines with different genetic background. So far we have obtained five different male sterile lines through backcrosses (Table 6). These

male-sterile lines were uniquely characterized by a high percentage of stigma exsertion, stronger tillering, good grain quality, resistance to diseases, etc.

Table 6. Development of TB-A male-sterile lines

Male sterility		Maintainer		Male-sterile lines
TB1A	×	TB3	→	TB9-1A
	×	T733-1	→	TB18-1A
	×	T733-2	→	TB18-2A
TB2A	×	TB6	→	TB13-2A
	×	TB1	→	TB17-2A

The different restoration-maintenance relationship between TB-A and WA-type male-sterile lines

A test focused on screening restorer lines was made while the development of sterile lines and maintainer lines was continued. The results of the primary crosses indicated that the maintainer line for WA-type sterile line was the maintainer line or partial restorer line for these new sterile lines, while the restorer line for the WA-type sterile line was the maintainer line or partial restorer line for line TB18-1 A. The above primary results were obtained by test crosses of F_1 of male-sterile line Zhenshan 97 A (WA-type) and 4 new male-sterile lines developed in this study with restorer lines (IR24, IR26, Minghui 63, 26 Zhazao, Fu 2-6, Test 64 and 1126) and maintainer lines (Zhenshan 97 B, II-32 B, Shaquenzao B, Kuangluai 4, T156 and D_3 B of the WA-type male-sterile lines (Table 7). It was proved that line TB18-1 A was the type of male-sterile line characterized by non-WA-type cytoplasmic-nucleic interaction, and there were much more restorers for new male-sterile lines than those for the WA-type male-sterile lines.

The heterosis of F_1 hybrids between TB-A male-sterile lines and some restorer lines

In comparison with the check varieties, F_1 hybrids between TB-A male-sterile lines and 7 restorers, were distinguished in the field by taller plant height, increased number of tillers per plant, grains per panicle and shorter growth duration. The average increase was 15.4 for the number of effective tillers, 29.0 for the number of grains per panicle, 0.5 g for 1000-grain weight, 12.7 cm for plant height and 4.4% for spikelet fertility, of which the increase in number of effective tillers and grains made more important contributions to the increase in grain yield (Table 8). We expect that the TB-A lines will play an important role in the production of hybrid rice in the near future.

Table 7. Comparison of the restoring-maintaining relationship between TB-A male-sterile lines and wild abortive (WA) male-sterile lines based on the fertility of F_1 generation

Male-sterile line		Restorer lines for WA type male-sterile lines							Maintainer lines for WA type male-sterile lines					
		IR24	IR26	Ming-hui 63	26 Zha-zao	Fu 2-6	Test	1126	Zhen-shan 97B	II 32B	Sha-quen-zao B	Kuang-luai 4	T1 156	D3B
WA type	Zhenshan 97A	44.8	49.0	61.6	62.0	88.2	56.1	70.5	0.6	0.7	2.6	2.2	5.9	0
TB-A type	TB18-1A	26.3	3.4	27.1	5.2	0	0	17.8	–	59.0	45.0	73	38.5	–
	TB13-2A	–	–	–	25.6	–	71.0	–	87.6	–	–	–	31.6	75.0
	TB9-1A	71.0	73.8	79.4	–	–	–	–	–	49.1	91.8	–	–	–
	TB18-2A	–	–	–	61.5	–	–	–	90.8	42.7	21.3	–	91.8	–

Table 8. Performance of F_1 hybrids of TB-A sterile lines with various restorers

Hybrids and varieties	Seed-setting rate		1000-grain weight		No. of effective tillers/plant		No. of grains /plant		Plant height	
	(%)	percentage of increase	(g)	percentage of increase	(no.)	percentage of increase	(no.)	percentage of increase	(cm)	percentage of increase
TB18-2A × T156	82.7	+4.4	26.3	–0.7	26	+7	139	+22	100	+15
T156	78.3		27.0		19		117		85	
TB18-2A × Milyang	81.3	+28.5	26.3	1.1	31	+10	125	+44	105	+15
Milyang	52.8		25.2		21		81		90	
TB18-1A × II-32B	61.9	+3.9	25.9	0.1	33	+20	142	+13	113	+3
II-32B	58.0		28.8		13		129		110	
TB9-1A × Teqing	67.5	+11.5	27.7	0.6	28	+8	202	+19	111	+6
Teqing	56.0		27.1		20		183		105	
TB18-2A × Zhenshan 97B	76.7	+15.1	26.0	0	21	+8	127	+22	127	+22
Zhenshan 97B	61.6		26.0		13		105		105	
TB13-2 × D3B	54.5	–20.0	28.3	+3.1	45	+20	111	+36	100	+15
D3B	74.5		24.9		25		75		85	
TB13-2A × Milyang	81.7	+20.2	24.6	–0.9	46	+27	109	+25	95	+10
Milyang	61.5		25.7		19		84		85	

Conclusions

As a result of the study which was initiated already in 1986, we can reach a conclusion that in vitro culture in combination with suitable doses of gamma rays radiation can increase the procentage of regenerated green plantlets of both *japonica* and *indica* rice. For somatic tissue culture, the radiation effect on redifferentiation and/or percentage of green plantlets formation is decreasing with the following order of callus treatment:

- treatment of callus with green spots,
- treatment of callus with developing shoots,
- treatment of germinating embryos.

Much more mutants appeared in the progeny of regenerated green plants derived from in vitro cultures treated with gamma-rays, in comparison with those from conventional irradiation or in vitro cultures. The mutants, which include those with desirable agronomic characters such as good grain quality, male-sterility and stigma exsertion, will supply the rice breeding programme and enrich available germplasms. With a view to broadening the genetic diversity of rice, the anther cultures from progenies of *indica* rice crosses in combination with mutagenesis will become an active and promising part of this integrated technique, as long as the efficiency of anther culture of *indica* rice will be increased.

In China, all the male-sterile lines up to now were developed by crossing (through wide hybridization, distant hybridization, etc.). In this study, using methods of biotechnology in combination with mutation techniques, male-sterile mutants (cytoplasm-nucleus interactive) also have been obtained. This indicates that a new approach to male-sterile line breeding has been established.

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