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Characteristics of genetic variation in the progenies of protoplast-derived plants of rice, *Oryza sativa* cv Nipponbare

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Abstract Genetic variation in protoplast-derived rice (*Oryza sativa* L.) plants was characterized using first and second generation selfed progenies. A total of 133 regenerated plants were obtained from ten protoplasts of the *japonica* rice cultivar Nipponbare. Sixty two regenerated plants which set enough seeds for the subsequent field tests at the next generation and were derived from five protoplasts were selected, and their selfed seeds were used as the first selfed-seed progeny (Pt_1 generation). Fifteen plants were selected from each of the 15 Pt_1 lines, and their selfed seeds were used for tests at the Pt_2 generation. Thirty seven Pt_1 lines (60%) segregated plants with detrimental mutant characters of yellow-green phenotype, dwarf stature, dense and short panicle, or low seed fertility. According to the segregation patterns in the lines having mutated plants among those originated from the same protoplasts, the stages of mutation induction were estimated. Additionally, five quantitative traits were changed in almost all Pt_1 and Pt_2 lines. Varied quantitative traits of heading date, number of spikelets per panicle, and seed fertility, were in a heterozygous state. However, culm and panicle lengths showed high uniformity, whereas reduced culm and panicle lengths were caused by mutational changes in polygenes and/or multiple genes.

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Introduction

The term 'protoclonal variation' has been used for the type of somaclonal variation found in regenerated plants after protoplast culture (Abdullah et al. 1989). The usefulness of somaclonal variation as a tool for the enlargement of genetic variation has been discussed by many workers (Shepard et al. 1980; Larkin and Scowcroft 1981; Evans and Sharp 1986; Ogura and Shimamoto 1991). However, when DNA is introduced into protoplasts, the occurrence of protoclonal variation is undesirable because of its disturbance in evaluating effects of foreign DNA expression. Therefore, it would be valuable, for both mutation breeding and gene introduction, to estimate the range of protoclonal variation and to identify the types of somaclonal mutations prior to the application of protoplast culture.

Previous studies on genetic variation in protoplast-derived rice plants gave contrasting results. The first study on protoclonal variation, reported by Ogura et al. (1987, 1989), showed that the agronomic characters of regenerated plants and their first selfed progeny from four cultivars were only slightly changed. Furthermore, they were phenotypically uniform and stable as compared with those of the original control plants. However, Abdullah et al. (1989) reported significant changes in quantitative characters in all of the protoplast-derived first progeny plants of rice.

Tissue culture-derived rice plants often possess mutated genes, and the mutations are generally thought to have been induced during callus growth and plant regeneration. Fukui (1983) reported that four mutations occurred in the 12 lines derived from a single callus of a rice seed, and identified the order of the stages at which each mutation was induced. However, the induction period of the mutations has not been studied in protoplast-derived rice plants.

In the present study, plants were regenerated from single protoplasts of a *japonica* rice cultivar, Nipponbare, and their first progeny lines which segregated mutants were examined to evaluate the order of the stages of mutation induction. Quantitative characters were also described by analyses of plants in the first and the second progenies of the same regenerated plants. Additionally, the nature of the mutations in quantitative traits of protoplast-derived plants is discussed.

Materials and methods

Primary calli were induced from mature seeds of a *japonica* rice (*Oryza sativa* L.) cultivar, Nipponbare, and suspended in liquid medium for 2 or 3 weeks. Protoplasts were isolated from suspended cells. Regenerated plants used in the present experiment were obtained from ten calli derived from single protoplasts. Procedures for protoplast culture and plant regeneration were as described by Kyozuka et al. (1987).

Table 1 shows the regenerated plants and their progeny lines examined. All the plants derived from a single protoplast were regarded as a member of a protoclonal family, which is denoted by the code numbers I to X.

The regenerated plants (hereafter denoted as the Pt_0 generation) were individually transplated to a paddy field of the Ishikawa Agricultural College in the summer of 1990. Morphological characteristics and selfed seed fertilities of the main panicles were examined.

Seeds obtained by self fertilization in respective Pt_0 plants which yielded enough seeds for the subsequent field tests were sown as lines in nursery boxes, along with those of the control cultivar, in the early spring of 1991. They were regarded as the Pt_1 generation. The seedlings were grown in a greenhouse and individually transplanted to the paddy field, at the stage of four or five leaves, in the middle of May. The frequencies of morphological and physiological variants were observed. Variation in four agronomic characters, culm length, panicle length, the number of spikelets per panicle and seed fertility, were measured using 28–50 plants in each line. Seedlings showing detrimental mutations, such as yellow-green leaf and dwarf stature, were not transplanted to the paddy field.

Fifteen plants in each of 15 Pt_1 lines in three protoclonal families were selected for tests at the Pt_2 generation. Plants from selfed seeds of each Pt_1 plant were designated as lines, giving 225 lines in the Pt_2 generation. They were sown in the nursery boxes and grown as in the previous year, and the seedlings were transplanted to the paddy field, according to a completely randomized block design with four replications, in the middle of May 1992. Each plot consisted of 28 plants and five randomly selected plants were used for the measurement of heading date, culm and panicle lengths, the number of spikelets per panicle and seed fertility. The data obtained were subjected to two-way analyses of variance.

Genetic analyses on culm and panicle lengths were carried out in 1994 using F_2 plants from crosses between three Pt_2 plants derived from three different protoclonal families and the original cultivar Nipponbare.

Results

Characteristics of the regenerated plants

A total of 133 regenerated plants were obtained from ten protoplasts of the rice cultivar Nipponbare (Table 1). They were grown in a paddy field for seed propagation and morphological observation. The seed fertilities of these regenerants varied from 2.7 to 77.5%.

All of the plants derived from protoclone III, four of five plants from protoclone IX, and 2 of 27 plants from protoclone X showed large leaves, thick stems, large and awned spikelets, and low fertilities ($< 20\%$). These traits were typical characteristics of polyploid rice plants (Ogura et al. 1987; Kanda et al. 1988; Guiderdoni and Chaïr 1992). Nakamura et al. (1994) showed that the spikelet traits provided a stable index of the ploidy level of rice plants. Thus, a total of 14 plants appeared to be caused by ploidy change. The difference in the frequency of the polyploid-like plants among the protoclonal families could be due to a difference in the period at which the cytogenetic change occurred. The high frequency of polyploid-like plants, such as those found in protoclones III and IX, suggested that the changes in chromosome number occurred in an earlier period of the division of protoplast-derived cells.

Selfed seeds obtained from 62 plants of five protoclones, I, IV, V, VIII and X, which yielded enough seeds, were used for subsequent field tests at the next generation.

Genetic variation in the Pt_1 generation

Thirty seven of sixty two Pt_1 lines (60%) segregated mutant plants in respect of four characters, yellow-green

Table 1 Pedigree relationships and number of lines of the protoplast-derived rice plants of *O. sativa* cv Nipponbare used for field tests

Protoclonal family	No. of regenerated plants obtained		No. of Pt_1 lines tested		No. of Pt_2 lines tested
I	11	select 8 plants	8	select 15 plants × 5 lines	75
II	4				
III	8				
IV	31	select 21 plants	21	select 15 plants × 5 lines	75
V	9	select 9 plants	9	select 15 plants × 5 lines	75
VI	12				
VII	16				
VIII	10	select 8 plants	8		
IX	5				
X	27	selectt 16 plants	16		
Total	133		62		225

Table 2 Number of Pt_1 lines of protoplast-derived rice plants which showed segregation in respect of four detrimental characters

Protoclonal family	No. of lines tested	Character			
		Yellow-green leaves	Dwarf stature	Dense panicle	Low seed fertility (< 60%)
I	8	0	0	0	1
IV	21	0	1	1	1
V	9	0	0	0	9
VIII	8	1	0	1	8
X	16	16	14	nt	nt

nt: not tested

leaves, dwarf stature, short and dense panicles, and low fertility (Table 2). The percentage of the plants showing mutated characters in each line ranged from 9 to 44%, suggesting that recessive mutations were induced during the in vitro culture and were then maintained in a heterozygous state in the Pt_0 plants.

Based on the segregation patterns in the lines giving rise to mutant plants, the induction period of the mutations during in vitro culture could be estimated. All of the lines in two protoclonal families, V and VIII, segregated plants with low seed fertility. This suggested that the isolated protoplast itself carried this mutation which was induced prior to the first division of the protoplasts giving rise to families V and VIII. Similarly, genetic changes leading to the yellow-green phenotype observed in all the lines in family X must have occurred before the first division of the protoplast. In family X, dwarf plants also appeared as segregants in 14 of 16 lines. Such a highly frequent mutation indicates that this was most likely induced earlier in protoplast culture. On the other hand, the variant phenotype which appeared in only one line in a family could have occurred late during the growth of the protoplast-derived calli or else during plant regeneration.

Three of five protoclonal families carried mutations which had occurred prior to the first division of the protoplasts. On the other hand, 6 of 62 lines possessed mutated genes which were generated late during the culture of protoplast-derived calli or during plant regeneration (Table 2). The frequency of the mutations generated at a later culture period was relatively low compared to that of the mutations pre-existing in isolated protoplasts.

All of the yellow-green and dwarf plants, and all the plants in protoclonal family X, were excluded from the measurement of agronomic characters.

Each of the 28–50 Pt_1 plants per line which were derived from the four protoclonal families I, IV, V and VIII were examined with respect to four agronomic characters (Table 3). Culms and panicles of the plants in all of the protoclonal families were shorter than those of the control plants. The number of spikelets per panicle was reduced in the Pt_1 plants. Seed fertility in four protoclonal families, especially V and VIII, was lower than that of the control. These data show that the mutations in the genes controlling agronomic and reproductive characters occurred with a high frequency.

Variation in the agronomic characters of Pt_2 plants

The effect of protoplast culture on agronomic characters was further examined using Pt_2 plants of the protoclonal families I, IV and V. Table 4 shows the performance of the Pt_2 plants as an average of the 15 lines which were the progeny of 15 randomly selected plants in the same Pt_1 lines. Variances between the 15 Pt_2 lines within the Pt_1 lines were also compared with that of 15 control lines, each of which was derived from plants of cv Nipponbare (Table 5).

The heading date differed with the protoclonal family. Pt_2 plants in protoclones I and IV showed a tendency for late heading. On the other hand, none of the lines in protoclone V showed any differences from the control. The variance between the Pt_2 lines was significantly larger than the control in 12 lines. This indicated that the number of genes controlling heading date were different between lines and were heterozygous in the previous generation. Culm and panicle lengths were reduced in all of the lines tested. Variances between the Pt_2 lines were not significantly larger than that of the control in almost all Pt_1 lines except for culm length in two and panicle length in one. The low levels of variation in these characters seemed to reflect high levels of

Table 3 Four agronomic characters of Pt_1 plants derived from four protoclonal families of rice

Protoclonal family	No. of Pt_1 lines tested	No. of plants measured	Character			
			Culm length (cm)	Panicle length (cm)	No. of spikelets per panicle	Seed fertility (%)
I	8	399	66.9 ± 3.9[c]	18.0 ± 1.3[c]	86.0 ± 14.4[c]	86.8 ± 8.5[c]
IV	21	1050	67.3 ± 3.8	18.1 ± 1.2	86.2 ± 13.8	87.6 ± 8.1
V	9	265[a]	71.1 ± 3.6	18.1 ± 1.0	87.5 ± 12.4	71.5 ± 23.5
VIII	8	400	68.8 ± 4.7	18.6 ± 1.1	87.7 ± 14.4	69.5 ± 23.8
Control		150[b]	77.6 ± 3.5	18.9 ± 1.1	96.3 ± 11.8	95.0 ± 3.5

[a] 90 plants were used for measuring culm and panicle length
[b] 20 plants were used for measuring spikelet number and seed fertility
[c] Mean ± standard deviation

Table 4 Performances for five agronomic characters of Pt_2 plants, shown as the average in each Pt_1 line, of three protoclonal families of rice

Protoclonal family	Pt_1 line	No. of Pt_2 lines tested	No. of Pt_2 plants measured	Character				
				Heading data (Aug.1 = 1)	Culm length (cm)	Panicle length (cm)	No. of spikelets per panicle	Seed fertility (%)
I	A	15	300	10.2*	70.3**	20.5**	104.7	85.2**
	B	15	300	9.9	67.0**	19.9**	89.8**	81.9**
	C	15	300	8.1**	68.9**	20.0**	94.2**	80.9**
	D	15	300	10.3**	68.9**	20.7**	109.2	86.7**
	E	15	300	9.8**	68.1**	20.1**	99.6**	80.7**
IV	A	15	300	10.7**	67.4**	20.1**	100.7**	84.0**
	B	15	300	10.2*	67.1**	19.1**	92.8**	85.2**
	C	15	300	10.3**	67.4**	20.0**	110.6	86.3**
	D	15	300	9.1	67.6**	19.5**	102.1**	88.7*
	E	15	300	10.7**	69.0**	20.3**	109.4	85.5**
V	A	15	300	9.6	68.4**	19.4**	96.3**	81.7**
	B	15	300	9.9	67.3**	19.1**	92.6**	79.7**
	C	15	300	9.9	69.3**	19.2**	91.1**	76.5**
	D	15	300	9.4	69.7**	19.4**	98.1**	77.7**
	E	15	300	9.7	69.8**	19.4**	93.5**	76.6**
Control		15	300	9.5	74.3	21.3	111.1	92.5
5% lsd				0.6	1.2	0.4	6.5	3.8
1% lsd				0.7	1.4	0.5	7.5	4.4

* and ** Significantly different from the control at 5 and 1% levels, respectively

Table 5 Mean squares in analyses of variance of five agronomic characters between the Pt_2 lines in each Pt_1 line of three protoclonal families of rice

Protoclonal family	Pt_1 line	*df*	Character				
			Heading date (Aug. 1 = 1)	Culm length (cm)	Panicle length (cm)	No. of spikelets per panicle	Seed fertility (%)
I	A	14	1.44**	3.24	0.16	24.3	11.56**
	B	14	1.69**	6.25*	0.64*	134.1**	37.21**
	C	14	0.81*	2.56	0.25	53.3*	14.44**
	D	14	1.69**	3.24	0.16	68.1*	4.00
	E	14	1.44**	2.56	0.25	22.5	9.61**
IV	A	14	1.21**	1.44	0.16	78.0**	9.00*
	B	14	1.00*	4.00	0.25	262.1**	15.21**
	C	14	1.44**	2.25	0.36	63.8*	7.84*
	D	14	0.64	1.69	0.16	19.8	4.00
	E	14	1.96**	2.56	0.09	44.8	8.41*
V	A	14	1.44**	1.96	0.25	27.5	112.4**
	B	14	1.00*	7.29*	0.25	157.0**	158.8**
	C	14	1.00*	4.41	0.25	46.2	141.6**
	D	14	0.49	5.76	0.16	28.3	158.8**
	E	14	0.64	3.24	0.16	38.4	166.4**
Control		14	0.25	1.96	0.16	16.4	2.25

* and ** Significantly larger than the control, after an *F*-test, at the 5% and 1% levels, respectively

homogeneity of the mutated genes. The number of spikelets of all the lines of protoclone V, and three lines of protoclones I and IV, were reduced. Seven groups of Pt_2 lines showed significantly larger variation than that of the controls, suggesting a high degree of heterogeneity between the Pt_2 lines. All Pt_2 lines, especially those in protoclone V, showed significantly lower seed fertilities than the control lines. Variations between the lines were significantly larger in almost all groups of lines, particularly in protoclone V, compared to the control. Only two groups of lines, I-D and IV-D, showed a small variation in fertility between the Pt_2 lines. These results suggest that the number of mutant genes concerned with seed fertility were different between the protoclones, and

the high degree of variation could be due to a high degree of heterogeneity of the genes involved.

In 225 Pt_2 lines examined, eight segregated plants having deterimental characters; namely, dwarf stature in five lines, a small number of culms in two lines, and dense and shorter panicle in one line. This indicates that 4% of Pt_1 plants of normal morphology had some detrimental recessive mutations in a heterozygous state.

Genetic analysis of short culm and panicle

To examine whether the variations in quantitative characters found in the Pt_2 generation were controlled by a polygenic system, three plants randomly selected from each of the three protoclonal families were crossed with the control cultivar, Nipponbare. The distribution of culm and panicle lengths was then studied in their F_2 plants. As shown in Fig. 1, as a typical case, all three F_2 populations showed a unimodal normal distribution for the two characters measured. These results indicated that protoclonal variation in culm and panicle lengths was induced by mutational changes in polygenes and/or multiple genes.

Discussion

In the present study, both continuous and discontinuous protoclonal variation was shown to be inherited by regenerated plants and their progenies from cell clones derived from single protoplasts. More than half of the regenerated plants showed detrimental effects, such as weakness, chlorophyll deficiency, dwarf stature and low fertility, and insufficient seeds were obtained to produce the next generation. Even at the succeeding generations of these regenerated plants which showed relatively higher seed fertilities and normal morphology, seed fertility had not recovered to the level of the control plants in the Pt_2 generation. Detrimental segregants frequently appeared in both the Pt_1 and Pt_2 generations. These results suggest that almost all regenerated plants from protoplast culture had more or less detrimental mutated genes. Our results are consistent with those of Abdullah et al. (1989), who showed a high frequency of mutations in agronomic characters in the first progeny of protoplast-derived rice plants, and of Kanda et al. (1988), who reported deleterious effects on the viability of the protoplast-derived regenerated rice plants after long-term culture of calli. However, one mutated character, short culm, might prove of value as a genetic resource through its introduction into plants which have a long culm and show weak lodging resistance.

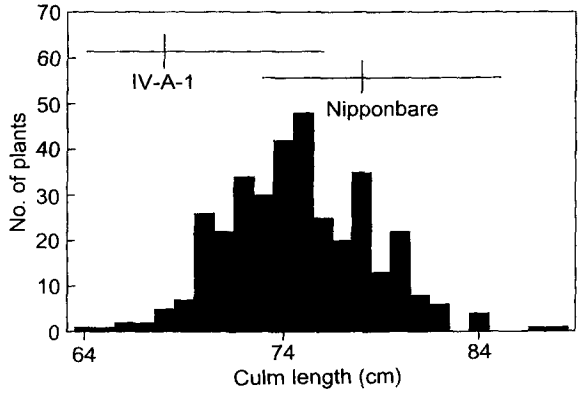
Brown et al. (1990), using RFLP analysis, detected a high degree of genetic instability among rice plants regenerated from protoplasts. The high level of DNA polymorphism observed might reflect the high frequency of phenotypic mutations, such as those found both in this study and that of Abdullah et al. (1989), although the molecular basis of these phenotypic changes is unknown.

The segregation patterns in the Pt_1 lines having mutants among protoclonal families (Table 2) revealed that these mutations were induced in the following order: (1) mutations causing low seed fertility in protoclones V and VIII, and a yellow-green phenotype in X, (2) the dwarf mutation in X, and (3) mutations concerning other characters which appeared in only one line of each family. These results indicate that mutations occurred at various times after callus induction and growth following protoplast isolation and until plant regeneration. These findings also suggest that mutation frequency increases with a prolonged culture period, as postulated by previous reports (Larkin and Scowcroft 1981; Armstrong and Phillips 1988; Nehra et al. 1992). Additionally, mutations occur more frequently prior to protoplast isolation than after cell division of the protoplasts. Therefore, there is higher probability that regenerated plants derived from the same protoplast will have the same mutant gene(s).

In the progeny of the regenerated plants derived from mesophyll protoplasts of lettuce (Engler and Grogan 1984), tobacco (Lörz and Scowcroft 1983) and *Nicotiana sylvestris* (Prat 1983), protoclonal variation was analyzed to distinguish the mutations which occurred prior to protoplast division and those which occurred after division of the protoplasts. All these authors reported that mutations occurred prior to protoplast division at a low frequency. The difference between their results and ours might be due to a difference in the plant material used for protoplast isolation. Mesophyll protoplasts were isolated directly from leaves, whereas the rice protoplasts used in our experiment were prepared from suspension cells grown in culture.

Ogura et al. (1989) reported that slightly changed quantitative traits were very uniform in the first progeny of protoplast-derived rice plants. However, the Pt_2 generation of the present experiment revealed that genes concerned with heading date, the number of spikelets

Fig. 1. Distribution of culm length in F_2 plants ($n = 355$) derived from a cross between a plant in Pt_2 line, IV-A-1, and cv Nipponbare. ⊣—⊢: means and ranges of the parents



per panicle, and seed fertility must have been in a heterozygous state in the Pt_1 generation, whereas culm and panicle lengths in most of the progeny lines showed uniformity. Quantitative traits in protoplast-derived progenies were significantly changed and, in most cases, showed segregation.

The mode of inheritance of single gene mutations observed in tissue culture-derived rice plants has been well analyzed (Fukui 1933; Sun et al. 1983; Oono et al. 1984; Ogura et al. 1988) However, genetic analysis of quantitatively affected characters induced by tissue culture have not yet been carried out. The present study showed that mutational changes in polygenes and/or multiple genes reduced culm and panicle lengths (Fig. 1). However, the reasons for the low degree of variance, and why all of the Pt_1 and Pt_2 lines had the same characteristics, are still unknown. Oono (1985) reported that putative homozygous dwarf mutants were obtained by seed callus culture of rice and that the phenotype of the mutants disappeared in the F_1 and F_2 generations. The mutants showing short culm and panicle lengths found in the present study were different from the homozygous mutants studied by Oono (1985), since the traits of culm and panicle lengths varied continuously in the F_2 populations.

A few mutant segregants appeared in 8 of 225 Pt_2 lines. Niizeki et al. (1989) and Cheng et al. (1992) also reported in rice anther culture and wheat embryo culture, respectively, that only one or a few mutated plants were found in lines derived from single regenerated plants. Cheng et al. (1992) suggested that this phenomenon resulted from intra-spike chimeric structures in regenerated plants and that mutations continued to occur after the formation of the primordial cells. The same mechanism might affect the distribution of occurrence of mutations in the panicles of regenerated rice plants, and its effects might have been reflected in the Pt_2 plants.

Both continuous and discontinuous mutant characters appeared in the first and second selfed progenies of protoplast-derived rice plants. The frequency of mutations was high, and most mutant characters segregated and were agronomically detrimental. Therefore, when protoploast culture is applied to mutation breeding, a large number of regenerated plants will be required to select beneficial mutant lines. For transformation, the high frequency of mutations is undesirable. Consequently, in order to reduce the frequency of mutations, microprojectile- and *Agrobacterium*-mediated methods (Christou et al. 1991; Cao et al. 1992; Hiei et al. 1994) may be more useful for rice transformation than the protoplast method because of the shortened tissue culture period involved. To confirm this, a comparative study on the somaclonal variation generated in protoplast-derived plants and in callus-derived plants will be necessary.

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