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Sequential occurrence of mutations in a growing rice callus

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Summary. Four mutations for early heading, albina, short culm and sterility were obtained in the progenies of twelve rice plants regenerated from a single callus of a rice seed. Studies on the segregation rates of these mutations revealed that for each mutation a single recessive gene was likely to be involved and that there was no linkage among the genes. The segregation pattern also showed that these mutations were induced in the following sequence: early heading, albina, and short culm and sterility during the stage of callus growth until the beginning of the regeneration of the rice plants.

Key words: Tissue culture – Mutation – Sequential mutations – Rice – *Oryza sativa* L.

Introduction

In recent years significant progress has been made in plant tissue culture. The potential practical contribution of tissue culture to plant breeding has been heralded by many authors (Scowcroft 1978; Maliga 1980). The utilization of new genetic variability induced either spontaneously or artificially during the culture process is one of the major objectives of tissue culture (Spiegel-Roy and Kochba 1977). Cell culture methods have opened up new possibilities for mutation breeding by making it possible to select mutants among large populations of cells (Chaleff and Parsons 1978).

Several biochemical mutations have been obtained by tissue culture in haploid tobacco (Carlson 1973; Maliga et al. 1973; Bourgin 1978; Chaleff and Parsons 1978), tobacco (Widholm 1974; Márton and Maliga 1975), soybean (Sung 1976), barley (Bright et al. 1979) and *Datura* (Schieder 1976). On the other hand, studies on mutations for morphological and physiological characters of practical importance obtained in tissue culture are rather few. Only a few authors have reported the occurrence of some mutations. For example, early and late heading, long and short culm, and sterility were observed in rice (Oono 1975; Chen and Li 1978), wheat (Chen and Li 1978) and oats (Cummings et al. 1976). It was also reported that more than two mutations for different traits were sometimes found among the progenies of one regenerated plant. These mutations have been considered to be induced and accumulated in growing calluses and/or regenerating plants. There is, however, no critical work on the stage of mutation induction or the mode of inheritance.

In this article evidence that four mutations were induced during the stage of callus growth is presented. It was also demonstrated that the four mutations for the characters early heading, albina, and short culm and sterility were independently induced in this sequence during the period of callus growth.

Materials and methods

Plant material

Twelve rice plants regenerated from a single callus and their selfed progenies were used throughout the experiment. The callus from which the twelve rice plants were regenerated was obtained out of over a hundred calli. Each callus was induced from seed culture of a cultivar, "Nipponbare", of rice (*Oryza sativa* L.).

Culture techniques

Sterilized brown rice was placed on N_6 agar medium (Chu et al. 1975) containing 2×10^{-5} M of 2,4-D and 2.5×10^{-4} M of *p*-fluorophenylalanine (PFP) adjusted to pH 5.8 before autoclaving. After two weeks, the calli induced were transferred for regeneration to the N_6 medium supplemented with 1×10^{-5} M of 6-benzyladenine (BA) and 5×10^{-9} M of 1-naphthalenacetic acid (NAA). The same period of culture as for callus induction was applied for plant regeneration. Then the materials were transferred to N_6 basal medium supplemented with the minor elements and the organic substances of Murashige and Skoog's medium. The whole culture was carried out in a room set at a temperature of 27 °C under 12-hour-day length.

Progeny test

The plantlets (T_1 generation) regenerated from a single callus, No. 100, were transplanted together with agar medium from a test tube to a pot in a greenhouse. After two weeks when the plantlets could become acclimatized, each of them was carefully separated and transplanted to new pols one by one. Twelve rice plants derived from the single callus survived and grew to maturity. Several characters, such as plant type, leaf color, heading date, pollen fertility, culm length, panicle length, number of panicles, flag leaf length and width, and seed fertility were examined. Each panicle of the T_1 plants was covered with a bag and self fertilized. All the panicles of each regenerated rice plant were harvested and a total of 114 lines from twelve T_1 rice plants were raised in applying the one panicle-to-one line method and maintained as sublines (T_2 generation) of each T_1 plant. A group of sublines derived from the same T_1 plant was designated as 'family' in this experiment. Thirty kernels of each sublines were sown on a nursery bed where germination rate and chlorophyll mutations were checked. About half of the seedlings of each subline were then transplanted in an experimental paddy field. The same characters as those of the T_1 generation except for flag leaf characters and pollen fertility were examined in the T_2 generation.

Screening of mutants

Evaluation of mutants for qualitative characters such as chlorophyll mutation was carried out in a nursery bed with the naked eye. Evaluation of mutants for quantitative characters such as heading date, culm length, and panicle length was carried out by comparing the distribution of each character in the progenies with the corresponding one in the controls. That is, the plants having a character(s) whose value(s) fell out of the confidence limit ($\alpha=0.01$) set up according to the character distribution in the controls were estimated to be mutants for the character(s). As for the sterility mutation, plants having over 25% of abortive kernels in a panicle of the plant were considered as mutants.

Results and discussion

Table 1 shows several characters of plants regenerated from the single callus, No. 100. Evaluation of each character except for plant type, number of panicles and leaf color were examined on the main culm. Heading dates of the twelve T_1 plants varied from August 25 to September 5. The mean value and the standard deviation were August 28.2 and 3.6, respectively. Culm length ranged from 61.0 cm to 79.0 cm and the mean value and the standard deviation were 68.8 and 5.1, respectively. Both pollen and seed fertility rates exceeded 90% on the average. Although plants No. 5 and No. 8 showed a fairly low fertility compared with the other plants, the rate of seed fertility of these plants reached about 60%. All the plants were found to be normal with regard to flag leaf length and width, leaf color and other characters. Therefore it was concluded that the regenerated plants were apparently normal so far as the characters examined were concerned.

In the next generation, however, mutations were observed for several characters among the progenies of the regenerated plants. The mutations obtained were as follows: albina in a nursery bed; and early heading, short culm and sterility in an experimental field. Table 2 shows occurrence of albino mutation in T_2 generation. 2884 seedlings were examined and 278 of the total number of albino seedlings were found in 44 sublines. All the sublines that segregated for albina belonged to the families of T_1 plants, No. 1, 2, 9, 10 and 11, whereas no albino seedlings was found in the other sublines of the families derived from T_1 plants No. 3, 4, 5, 6, 7, 8 and 12. It was worth noting that all the 44

Table 1. Characteristics of the twelve plants regenerated from the single callus, No. 100

Plant No.	Heading date	Culm length (cm)	Panicle length (cm)	No. of panicles	Flag leaf (cm)		Fertility (%)		Leaf color	Plant type
					Length	Width	Pollen	Seed		
1	Aug. 25	79.0*	20.0	7	27.8	1.05	81.05	95.24	normal	normal
2	Aug. 25	67.0	19.5	8	37.0	1.40	100.00	96.00	normal	normal
3	Aug. 29	72.0	18.5	12	25.2	1.30	98.20	97.46	normal	normal
4	Aug. 25	74.0	17.5	8	19.1	1.25	99.32	98.48	normal	normal
5	Sept. 5*	61.0	16.0	16*	23.5	1.20	97.80	59.92*	normal	normal
6	Aug. 26	63.0	15.0	9	14.5	0.95	89.47	97.20	normal	normal
7	Aug. 26	68.0	22.0	11	34.5	1.30	98.51	98.38	normal	normal
8	Aug. 27	65.0	16.0	7	17.0	1.00	39.53**	60.90*	normal	normal
9	Aug. 31	71.0	19.5	9	40.0	1.35	99.21	97.24	normal	normal
10	Aug. 31	66.0	20.0	9	38.0	1.30	94.38	93.68	normal	normal
11	Aug. 25	67.0	19.0	11	28.7	1.35	95.52	95.92	normal	normal
12	Sept. 1	73.0	19.5	7	23.8	1.30	97.37	94.48	normal	normal
Mean	Aug. 28.2	68.8	18.5	9.5	27.4	1.23	90.86	90.41	–	–
SD	3.6	5.1	2.0	2.7	8.5	0.15	17.04	14.09	–	–

*, ** Significant at the 5% and 1% level, respectively

Table 2. Emergence of chlorophyll mutation in the progenies of T_1 plants

T_1 plant No.	No. of lines tested	Total no. of seeds sown	Germination rate[a] (%)	No. of lines segregating albina	No. of albina	Total no. of plants tested	Mutation rate (%)	χ^2 (3:1)
1	7	210	84.76	7	51	178	28.65	$P > 0.25$
2	8	240	82.08	8	43	197	21.83	$P > 0.25$
3	12	360	92.78	0	0	334	0.00	
4	8	240	88.75	0	0	213	0.00	
5	16	371	79.18	0	0	282	0.00	
6	9	270	89.26	0	0	241	0.00	
7	11	330	92.27	0	0	306	0.00	
8	7	210	85.71	0	0	180	0.00	
9	9	270	92.59	9	58	250	23.20	$P > 0.50$
10	9	268	87.50	9	58	239	24.27	$P > 0.75$
11	11	328	82.42	11	68	272	25.00	$P \gg 0.90$
12	7	210	91.43	0	0	192	0.00	

[a] Figures are given in average of the germination rate of each line derived from the same T_1 plant

sublines derived from five T_1 plants segregated for the albino seedlings without exception as shown in Table 2, suggesting that the T_1 plants No. 1, 2, 9, 10 and 11 possessed the albino gene. Since the leaf color was normally green in the T_1 generation, it can be considered that the albino gene was reccessive. The results of χ^2-test showed that all the segregation ratios closely fitted the expected 3 : 1 ratio, indicating that the albino gene is a single recessive one. Analysis of the segregation pattern of the albina also enabled determination of the induction stage of the mutation. The fact that the five plants regenerated from different parts of a callus had the albino mutation unlike the others suggests that the callus itself had a heterogeneous constitution for the albino gene. Namely, an albino mutation had been induced in a cell of the growing callus and made a sector of cells with albino gene before the beginning of the differentiation of the initial cells of all five plants. It is very difficult to postulate that the albino mutations might have been induced in every initial cell of the five plants or 44 panicles of each plant, because the mutation frequency is usually very low. As for the germination rate, most of the sublines had a germination rate exceeding 80% and no abnormality was found.

After transplantation of green seedlings in the experimental field, mutations for both physiological and morphological characters were observed. Table 3 shows the mutation frequencies for heading date, culm length and sterility. It is obvious that early heading, short culm and sterile mutations were induced among T_2 progenies. Each mutation frequency for heading date, culm length and sterility was calculated by adding the total number of mutants induced in each family derived from the same T_1 plant. Indeed the same tendency, namely the fact that the same mutations were commonly induced among the T_2 sublines in a family, was also observed in the mutation for each character though this tendency was somewhat less distinct in quantitative characters such as heading date and culm length compared with the chlorophyll and the sterile mutations. According to the induction frequency of mutants among the T_2 progenies, families were divided into two groups. As for early heading, for example, mutation frequencies were much higher in the families of T_1 plants No. 1, 2, 5, 6, 7, 8, 9, 10 and 11 than in the families of the remaining plants. The average mutation frequency in the families with a higher mutation frequency was 14.01% and 3.65% in those with a lower mutation frequency (significant at 1% level). This tendency was also observed in the mutations for short culm and sterility. In the case of the short culm mutation, the group with a higher mutation frequency included the families of T_1 plants No. 1, 2, 10 and 11, and that with a lower mutation frequency included No. 3, 4, 5, 6, 7, 8, 9 and 12. Average mutation frequency was 13.03% for the higher group and 3.08% for the lower one (significant at 1% level). These results completely agree with those obtained in the sterile mutation and the average mutation frequency in the higher group was 33.25% compared with 1.64% in the lower one (significant at 1% level). The results of Table 3 also indicate that there was no relationship between the T_1 plants with low fertility and the T_1 plants segregating a large number of sterile mutants in their progenies. This means that the low fertility observed in the T_1 generation must be ascribed to a change which is not heritable, such as physiological damage caused in the process of tissue culture.

Mutations for three characters could not be observed among the T_1 plants and the sterility of the T_1 generation was not associated with the mutation in the T_2 generation. Although the T_1 plants differed from

Table 3. Segregation frequency of mutant[a] for three characters in T_2 generation

T_1 plant No.	Heading date (%)		Culm length (%)		Sterility (%)	No. of tested	
	Early	Late	Short	Long		Lines	Plants
Control	0.00	0.45	0.45	0.00	0.00	14	220
1	15.84	0.00	14.85	0.00	21.78	7	101
2	6.50	0.00	10.66	0.00	45.90	8	123
3	1.60	0.53	4.81	0.00	1.60	12	187
4	5.65	0.00	6.40	0.00	1.61	8	125
5	25.37	0.00	2.93	0.00	5.37	16	205
6	11.51	0.72	2.16	0.00	1.44	9	139
7	12.87	0.58	3.51	0.00	1.17	11	171
8	12.04	0.00	2.78	0.93	0.00	7	108
9	18.12	0.72	0.00	0.72	0.72	9	138
10	10.07	0.00	15.83	0.00	30.94	9	139
11	13.77	0.00	10.78	0.00	28.74	11	167
12	3.70	0.93	2.78	0.00	0.93	7	108

[a] Plants having the character which falls outside the confidence limits ($\alpha = 0.01$)

each other in the genetic constitution of the mutant gene(s) as revealed in T_2 generation, they did not show significant changes in both morphological and physiological characters among the T_1 plants. Such findings indicate that these mutations did not influence the expression of the characters and the viability of the T_1 plants. It was thus concluded that the genes which were involved in the mutations for the characters were recessive like the albino gene and must be present under heterogeneous conditions in the T_1 generation.

The induction of mutations among the T_1 plants is indicated in Table 4. T_1 plants numbers were rearranged in Table 4 based on the segregation pattern of the mutations. In this table the T_1 plants which segregated in a proportion of over 10% of the mutants for each character in the T_2 generation were considered to have been the latent mutants for the character. The mutation rate for early heading of the T_1 plant No. 2 was somewhat low due to occasional fluctuations in the field experiments and small population size, however it still showed the highest mutation rate among the remaining T_1 plants. The results of Table 4 show that mutations for early heading, albina and both short culm and sterility were not the expression of the pleiotropism of a single mutant gene because of the difference in the segregation pattern among the mutants. Mutations for short culm and sterility induced in the progenies of T_1 plants No. 1, 2, 10 and 11 showed the same segregation pattern. Segregation pattern of short culm and sterile mutants in each family which segregated both the mutants, however, revealed that there was no pleiotropy of a single gene, since the

Table 4. Cross index for T_1 plants and the characters mutated

Mutational group No.	T_1 plant No.	Characters[a]			
		Early heading	Albina	Short culm	Sterility
I	1	M	M	M	M
	2	M	M	M	M
	10	M	M	M	M
	11	M	M	M	M
II	9	M	M	N	N
III	5	M	N	N	N
	6	M	N	N	N
	7	M	N	N	N
	8	M	N	N	N
IV	3	N	N	N	N
	4	N	N	N	N
	12	N	N	N	N

[a] M: mutated, N: normal

sterile mutants were observed in almost all the classes of culm length. Therefore it is concluded that there is no mutant gene which has pleiotropic effect for the four mutant characters observed. According to the standards set up, the T_1 plants were divided into two major groups: the first one that included the plants which did not display mutatins in any character examined, such as the T_1 plants No. 3, 4 and 12, and the other to which belonged the group of mutants. Segregation pattern of mutations was, however, quite different among the mutants. T_1 plant No. 1 segregated for mutations for heading date, culm length, sterility and chlorophyll in the families, while T_1 plant No. 5 only for heading date. Then the T_1 plants were again divided into three subgroups as shown in Table 4. Plants No. 1, 2, 10 and 11 were mutants for all the characters listed on the table. Plants No. 9; and No. 5, 6, 7 and 8 were mutants for heading date and chlorophyll; and heading date only, respectively. Also the idea that the mutations of the regenerated plants were induced before the beginning of the differentiation of each plant would agree well in the case of these mutations in with the segregation pattern of the mutants, as discussed before. Mutations appeared to have been induced after the beginning of callus growth from the rice seed because some of the regenerated plants failed to show any mutation in their progenies and back mutation was known to be extremely rare. Since these regenerated plants were obtained from different parts of the callus, it is evident that the callus itself was composed of sectors consisting of cells with different mutant gene(s). The formation of genetic chimerism in a single callus as revealed by progeny analysis is considered to have been due to the induction of mutations in the growing callus.

One of the conspicuous characteristics shown in Table 4 is that the number of mutated characters decreased stepwise from group I to group IV. Figure 1 shows a schematic representation of the sequential induction of mutations in a growing callus. This could explain the mutation combinations that might reflect the order of the induction of mutations shown in Table 4. Namely, one or a few initial cells in the embryo of a rice kernel could have begun to form the callus and thereafter the mutation for early heading might have been induced at first in a certain cell of the callus so as to form a sector with mutation associated with the callus growth. A mutation for albina might have been then induced in a cell of a sector with the mutation of heading date. By the induction of two mutations the callus could have been divided into three kinds of sectors, 1) normal, 2) with mutation for early heading, and 3) with mutations for early heading and albina. Thereafter two mutations for short culm and sterility might have been induced in a cell of the sector with two mutations simultaneously or not. It was not possible to determine which mutation was first induced by analysing the segregation pattern of the mutants in the progenies, since the two mutations showed an identical segregation pattern among the regenerated plants obtained so far. After the four kinds of sectors had developed in a callus, the differentiation and development of the rice plants would have taken place. Another explanation, for example, that the four mutations might have occurred at the same time in a cell followed by back mutations induced successively along with callus growth, or that the three kinds of cluster mutations including one to four mutations might have occurred independently in different parts of the callus, seems improbable, since usually the mutation rate is low and the rate of back mutations is even very much lower except for special cases.

The effect of PFP, which was added in the medium for dedifferentiation, is difficult to evaluate. It has been reported that PFP might cause chromosome deletion in

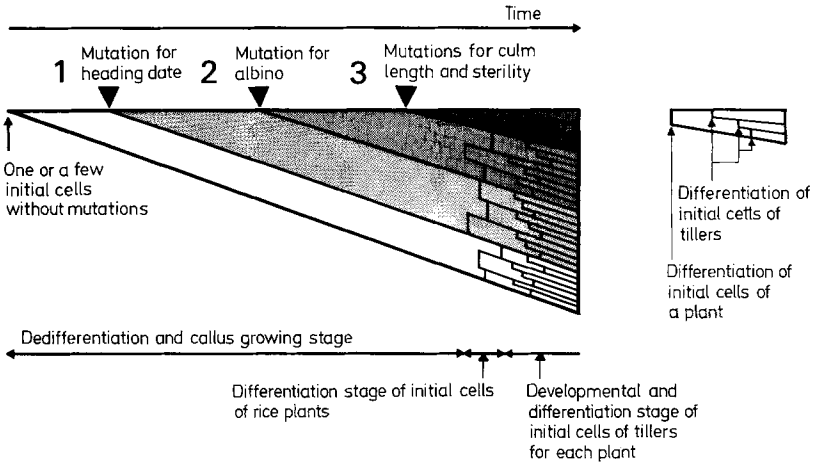

Fig. 1. Schematic representation of the sequential occurrence of the four mutations in a growing rice callus

the somatic cells of higher plants (Niizeki 1977) and promote the induction of mutations (Fukui and Kobayashi 1981). Then it might also be that in the current experiments the mutations were stimulated by PFP, although further studies should be carried out to substantiate such a hypothesis.

On the basis of the results obtained, it is concluded that the four mutations such as those for early heading, albina, and short culm and sterility were induced independently in this sequence and that the induction of the mutations occurred during the period of growth of the rice callus. Further genetic analysis of these mutants and of the mutations for other traits are now under way and will be reported elsewhere.

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