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Relationship between Callus Size and Plant Regeneration in Rice (*Oryza sativa* L.) Anther Culture

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Abstract
Relationship between callus size and plant regeneration in rice anther culture is described. There was no relation between the callus size and green plant regeneration. Regeneration of albino plants increased in proportion to the callus size in Nipponbare. The number of haploid green plants regenerated from calluses increased when the callus was > 2.0 mm in diameter. Spontaneous doubling of the chromosome number of green plants was promoted when the callus was 1.0-1.2 mm in diameter. The ratio of green plant regeneration (green plant/albino plant) and spontaneous doubling frequency of the chromosome number of green plants were higher in Akenohoshi than in Nipponbare, which may be related to the fact that the callus sizes of Akenohoshi were relatively small compared with those of Nipponbare. The effect of low temperature treatment of anthers on callus formation, regeneration of green and albino plants from calluses, and spontaneous doubling of the chromosome number of regenerated plants was also described. Low temperature treatment of anthers resulted in the increase of the callus formation frequency in Nipponbare, but not in Akenohoshi, as well as the increase of green plant regeneration and decrease of spontaneous doubling of the chromosome number of green plants, while low temperature treatment did not affect albino plant regeneration.

Discipline: Biotechnology
Additional key words: albino plant, green plant, ploidy level, pretreatment

Introduction

Rice tissue culture has been applied for rice improvement. Especially, anther culture has been applied to develop new varieties as a simple and time-saving method. Also, transformation and mutation have been applied to introduce new characters in diploid cells. By these methods the gene can be introduced into only one chromosome. As a result, the production of homozygous plants can not be achieved directly. Therefore, the development of a new cultivar through these methods is time-consuming or laborious in the same way as through ordinary breeding methods. On the contrary, transformation and mutation of haploid cells followed by chromosome doubling and plant regeneration enable to develop homozygous plants.

Calluses derived from rice anthers show various ploidy levels ranging from haploidy to high polyploidy, even if they are not treated by chemicals for doubling the chromosome number[1], and plants with various ploidy levels can be obtained[3]. Spontaneous doubling of the chromosome number is preferable to the production of new varieties by directly using anther culture of F_1, or progenies of F_1. However, spontaneous doubling before gene induction or mutagen treatment is not preferable to the transformation or mutation methods of haploid cells of anther. Therefore, if green plants can be obtained efficiently and the doubling of the chromosome number can be controlled, anther culture could be used for the rapid development of true breeding lines of rice.

There have been several reports dealing with the relation between the callus size and regeneration of green and albino plants in rice[5,7]. The problem is to determine whether the callus size is related to the spontaneous doubling of the chromosome number.

In the present paper, mainly the relationship

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between the callus size and plant regeneration was described in rice anther culture. The effect of low temperature treatment of anthers on callus formation and plant regeneration was also outlined.

Materials and methods

Oryza sativa L. cultivars, Nipponbare (*japonica* cultivar) and Akenohoshi (cultivar derived from F_1 sexual cross between *japonica* and *indica*) were used. They were grown in a glasshouse during the summer of 1992. Panicles were harvested when the base of the flag leaf was 2–4 cm above the base of the next lower leaf. The panicles were not sterilized.

N_6 medium[2], modified to contain 0.25 mg/l $Na_2MoO_4 \cdot 2H_2O$, 0.025 mg/l $CuSO_4 \cdot 5H_2O$, 0.025 mg/l $CoCl_2 \cdot 6H_2O$ and 100 mg/l myo-inositol, supplemented with 10^{-5} M dichlorophenoxyacetic acid (Exps. 1, 2, 3, 4) or α-naphthaleneacetic acid (NAA) (Exp. 5) and 3% sucrose, was used for callus formation. The pH of the media was adjusted to 5.8 with KOH and 0.8% (Exps. 1, 2, 3, 5) or 1.0% (Exp. 4) agar was added, then the media were autoclaved at 1 kg/cm^2 and 121°C for 15 min. Twenty anthers were placed in a dish 9 cm in diameter (15 ml medium). Anthers on the medium were treated at 10°C (Exps. 1, 2, 3, 4, 5) and 25°C (as a control, only Exp. 1) in the dark for 10 days after placement. They were then cultured at 25°C in the dark. Hundred and forty anthers were cultured for each treatment per cultivar. Callus formation frequency was expressed as the percentage of anthers that formed calluses after 40 days of culture (including duration of low temperature treatment of anthers).

For plant regeneration, N_6 modified medium containing 1% agar without hormones (15 ml in a 9 cm diameter dish) was used and incubation was conducted at 25°C under continuous illumination with white fluorescent light (20 $\mu Es^{-1}m^{-2}$). After 40 days of culture (including duration of low temperature treatment of anthers), 7–10 pieces of calluses, more than 0.8 mm in diameter, were transferred to the regeneration medium in a 9 cm petri dish. The plant regeneration frequency was expressed as the percentage of regenerated calluses after 40 days of culture.

After 40 days of culture for regeneration, plantlets, more than 10 cm in height, were transplanted into pots containing soil. Plantlets, less than 10 cm, were again transferred to the regeneration medium, and they were then transplanted to pots if their plant height exceeded 10 cm. The regenerated plants were grown in a glasshouse in the winter of 1992–1993.

The ploidy levels of the green plants regenerated from anther-derived calluses were identified based on their morphological characteristics[3,4] from November 1992 to April 1993 as follows: the plants that were shorter with a smaller glume and sterile, as haploids; the plants which had the same morphology and fertility as those of the original cultivars, as diploids (doubled haploids); the plants with larger glume, often having an awn, fertile or sterile, as polyploids or aneuploids. Frequency of haploid, doubled haploid, and polypoid + aneuploid plants was estimated as follows:

(number of calluses producing haploid, diploid, and polyploid + aneuploid plants, respectively) /(number of calluses regenerating green plants) × 100.

Results and discussion

Low temperature treatment of anthers of Nipponbare was effective for callus formation which was 1.9 times more active than that of the control. In the anthers of Akenohoshi, no effect of the treatment was observed (Fig. 1). Hundred and one varieties of *japonica* and fifty-four *indica* varieties were tested (unpublished data). The effect of the treatment was pronounced in all the *japonica* varieties, but not appreciable or not observed in *indica* varieties. Therefore, the *indica* traits may be responsible for the lack of the effect of low temperature treatment on callus formation in Akenohoshi.

Low temperature treatment of anthers increased the percentage of regeneration of green plants from calluses, while no effect of the treatment on the percentage of albino plant regeneration was observed (Table 1). Increase in the percentage of green plant

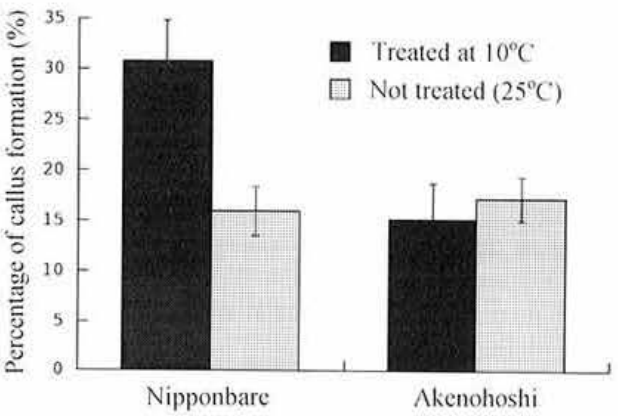

Fig. 1. Effect of low temperature treatment of anthers on the formation of calluses from rice anthers Bars indicate standard errors.

Table 1. Relationship between callus size and regeneration of green and albino plants

Cultivars	Treatment of anthers		Callus diameter (mm)	No. of calluses tested	No. of calluses producing plants			Percentage of calluses producing plants		
					Green	Albino	Plant[a)]	Green	Albino	Plant[a)]
Nipponbare	Low temp. (10°C)	Exp. 1	< 1.0	14	3	1	5	21.4	7.1	35.7
			1.0–1.2	49	6	12	20	12.2	24.5	40.8
			1.3–2.0	30	13	8	21	43.3	26.7	70.0
			2.0 <	23	9	7	16	39.1	30.4	69.6
			Total	116	31	28	62	26.7	24.1	53.4
		Exp. 2	< 1.0	50	8	8	17	16.0	16.0	34.0
			1.0–1.2	50	11	13	26	22.0	26.0	52.0
			1.3–2.0	49	7	15	22	14.3	30.6	44.9
			2.0 <	40	6	16	23	15.0	40.0	57.5
			Total	189	32	52	88	16.9	27.5	46.6
		Exp. 3	< 1.0	62	16	17	33	25.8	27.4	53.2
			1.0–1.2	81	26	27	55	32.1	33.3	67.9
			1.3–2.0	81	17	39	61	21.0	48.1	75.3
			2.0 <	13	5	4	9	38.5	30.8	69.2
			Total	237	64	87	158	27.0	36.7	66.7
		Exp. 1 + Exp. 2 + Exp. 3	< 1.0	126	27	26	55	21.4	20.6	43.7
			1.0–1.2	180	43	52	101	23.9	28.9	56.1
			1.3–2.0	160	37	62	104	23.1	38.8	65.0
			2.0 <	76	20	27	48	26.3	35.5	63.2
			Total	542	127	167	308	23.4	30.8	56.8
	Control (25°C)	Exp. 1	< 1.0	9	2	2	5	22.2	22.2	55.6
			1.0–1.2	21	3	4	8	14.3	19.0	38.1
			1.3–2.0	31	3	10	16	9.7	32.3	51.6
			2.0 <	23	5	9	16	21.7	39.1	69.6
			Total	84	13	25	45	15.5	29.8	53.6
Akenohoshi	Low temp. (10°C)	Exp. 1	< 1.0	23	2	2	5	8.7	8.7	21.7
			1.0–1.2	46	10	3	14	21.7	6.5	30.4
			1.3–2.0	9	2	1	3	22.2	11.1	33.3
			2.0 <	7	1	1	2	14.3	14.3	28.6
			Total	85	15	7	24	17.6	8.2	28.2
	Control (25°C)	Exp. 1	< 0.9	38	3	3	6	7.9	7.9	15.8
			1.0–1.2	96	6	11	19	6.3	11.5	19.8
			1.3–2.0	44	3	3	6	6.8	6.8	13.6
			2.1<	25	2	2	4	8.0	8.0	16.0
			Total	203	14	19	35	6.9	9.4	17.2

a): Plant = Green plants + Albino plants + Dead plants.

regeneration in the low temperature treatment of anthers is in agreement with the report of Zhou and Cheng[8)]. Akenohoshi exhibited high ratios of green plant regeneration (green plant/albino plant ratios were 2.15 in the low temperature treatment and 0.73 in the absence of treatment, while those of Nipponbare were 0.74 and 0.52, respectively).

Table 1 also shows the relationship between the callus size and percentage of plant regeneration. The percentage of green plant regeneration from calluses, less than 1.2 mm in diameter, was low under low temperature treatment of anthers of Nipponbare (Exp. 1). However, the results were not reproducible in repeated experiments (Exps. 2, 3). As the callus size increased up to 2.0 mm in diameter, the percentage of regeneration of albino plants increased. In Akenohoshi, the relationship between the callus size and plant regeneration was not clearly revealed

because most of the calluses were less than 1.2 mm in diameter. Wakasa[7] and Ozaki[5] reported that smaller calluses displayed higher abilities of green and albino plant regeneration. These observations were different from the data reported in the present paper. The size of the calluses used by the former authors was relatively large (diameters reported by Wakasa[7] ranged from 2–4 mm and 5–10 mm; those reported by Ozaki[5] were in the range of <2 mm, 2–4 mm and >4 mm). The calluses they used would be too large to observe a relationship between the callus size and plant regeneration ability since large calluses are old, which results in the reduction of cellular activity and induces a decrease of the regeneration ability of green and albino plants.

Haploid regeneration frequencies increased by low temperature treatment of anthers in Nipponbare and Akenohoshi (Table 2). When anthers were not

Table 2. Relationship between callus size and ploidy level of regenerated plants

Cultivars	Treatment of anthers		Callus diameter (mm)	No. of calluses tested	No. of calluses producing plants[a]			Percentage of calluses producing plants		
					HP	DHP	PAP[b]	HP	DHP	PAP[b]
Nipponbare	Low temp. (10°C)	Exp. 1[c]	< 1.0	8	5	3	0	62.5	37.5	0.0
			1.0–1.2	17	8	8	4	47.1	47.1	23.5
			1.3–2.0	34	21	15	1	61.8	44.1	2.9
			2.0 <	13	11	5	0	84.6	38.5	0.0
			Total	72	45	31	5	62.5	43.1	6.9
		Exp. 4	< 1.0	9	9	1	0	100.0	11.1	0.0
			1.0–1.2	22	14	10	2	63.6	45.5	9.1
			1.3–2.0	3	2	1	0	66.7	33.3	0.0
			2.0 <	0	–	–	–	–	–	–
			Total	34	25	12	2	73.5	35.3	5.9
		Exp. 5	< 1.0	7	4	3	0	57.1	42.9	0.0
			1.0–1.2	13	7	4	2	53.8	30.8	15.4
			1.3–2.0	14	9	5	1	64.3	35.7	7.1
			2.0 <	12	9	1	3	75.0	8.3	25.0
			Total	46	29	13	6	63.0	28.3	13.0
		Exp. 1 + Exp. 4 + Exp. 5	< 1.0	24	18	7	0	75.0	29.2	0.0
			1.0–1.2	52	29	22	8	55.8	42.3	15.4
			1.3–2.0	51	32	21	2	62.7	41.2	3.9
			2.0 <	25	20	6	3	80.0	24.0	12.0
			Total	152	99	56	13	65.1	36.8	8.6
	Control (25°C)	Exp. 1	< 1.0	2	1	2	0	50.0	100.0	0
			1.0–1.2	3	0	3	0	0	100.0	0
			1.3–2.0	3	1	2	0	33.3	66.7	0
			2.0 <	4	1	3	0	25.0	75.0	0
			Total	12	3	10	0	25.0	83.3	0
Akenohoshi	Low temp. (10°C)	Exp. 1	< 1.0	1	1	0	0	100.0	0	0
			1.0–1.2	10	1	8	4	10.0	80.0	40.0
			1.3–2.0	1	1	1	0	100.0	100.0	0
			2.1 <	0	–	–	–	–	–	–
			Total	12	3	9	4	25.0	75.0	33.3
	Control (25°C)	Exp. 1	< 1.0	3	1	2	1	33.3	66.7	33.3
			1.0–1.2	4	0	2	3	0	50.0	75.0
			1.3–2.0	1	0	1	0	0	100.0	0
			2.0 <	0	–	–	–	–	–	–
			Total	8	1	5	4	12.5	62.5	50.0

a): Plants with two or three ploidy levels were regenerated in some calluses.
b): HP; Haploid, DHP; Doubled haploid, PAP; Polyploid or Aneuploid.
c): Results of Exp. 1 in low temperature treatment of Nipponbare included the results of Exp. 2 and Exp. 3.

subjected to the low temperature treatment, doubled haploid frequency increased in Nipponbare and the frequency of regeneration of polyploid + aneuploid plants increased in Akenohoshi (Table 2). In these results, low temperature treatment of anthers is considered to suppress the spontaneous doubling of the chromosome number, which is in agreement with the observation of Tsay and Chen[6]. Spontaneous doubling of the chromosome number occurred more frequently in Akenohoshi than in Nipponbare.

Table 2 also shows the effect of the callus size on the ploidy levels of regenerated plants. In Nipponbare, haploid regeneration frequency was low in smaller calluses (1.0–1.2 mm in diameter) but increased in larger calluses (>2.0 mm in diameter) (Exp. 1). On the contrary, the doubled haploid regeneration frequency was high in smaller calluses (1.0–1.2 mm in diameter) and decreased in larger calluses (>2.0 mm in diameter) (Exp. 1). The same relationship was observed when 1.0% agar was used instead of 0.8% agar (Exp. 4) or when NAA was used as the hormone (Exp. 5). In total, in the low temperature treatment of anthers of Nipponbare, haploid regeneration frequencies were 55.8% when calluses 1.0–1.2 mm in diameter were used and 80.0% when calluses >2.0 mm in diameter were used; doubled haploid regeneration frequencies were 42.3 and 24.0%, respectively. In Akenohoshi, spontaneous doubling of the chromosome number mostly occurred in regenerated plants: regenerated plants were mostly derived from calluses 1.0–1.2 mm in diameter. These observations may be ascribed to the fact that a callus with a slow growth shows a high potential of spontaneous doubling of the chromosome number, or that a callus with late generation displays a high potential. Further studies are required to clarify these aspects.

In conclusion, when smaller calluses (1.0–1.2 mm in diameter) were transferred to a regeneration medium, doubled haploid plants could be obtained efficiently. If larger calluses (>2.0 mm in diameter) are transferred to a medium, haploid plants can be easily obtained.

References

1) Chen, C. C. & Chen, C. M. (1980): Changes in chromosome number in microspore callus of rice during successive subculture. *Can. J. Genet. Cytol.*, **22**, 607–614.
2) Chu, C. C. et al. (1975): Establishment of an efficient medium for anther culture of rice through comparative experiments on the nitrogen sources. *Sci. Sin.*, **17**, 657–668.
3) Nishi, T. & Mitsuoka, S. (1969): Occurrence of various ploidy plants from anther and ovary culture of rice plant. *Jpn. J. Genet.*, **44**, 341–346.
4) Oono, K. (1975): Production of haploid plants of rice (*Oryza sativa*) by anther culture and their use in breeding. *Bull. Natl. Inst. Agric. Sci.*, D**26**, 139–222 [In Japanese with English summary].
5) Ozaki, K. (1986): Effects of each factor to callus forming rate and regeneration rate in rice anther culture. 2. Effects of each factor to regeneration rate. *Kinki Chugoku Agric. Res.*, **71**, 27–31 [In Japanese].
6) Tsay, H. S. & Chen, L. J. (1984): The effects of cold shock and liquid medium on callus formation in rice anther culture. *J. Agric. Res. China*, **33**(1), 24–29.
7) Wakasa (1982): Application of tissue culture in plant breeding. Method improvement and mutant production. *Bull. Natl. Inst. Agric. Sci.*, D**33**, 121–200 [In Japanese with English summary].
8) Zhou, X. T. & Cheng, Q. L. (1981): The cold-pretreatment effect on the rate of pollen-induced plant of hsien rice. *J. Fujian Agric. College*, **3**, 25–32.

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