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Comparison of Somaclonal Variation between Two Regeneration Methods in Rice (*Oryza sativa* L.)

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The occurrence of somaclonal variations in rice was first reported together with the successful plant regeneration from callus[1]. In regenerated rice, earlier heading date, shorter plant height, and lower seed fertility are well known as phenotypic variation in agronomic characters[2-4]. The somaclonal variation has been perceived as an additional and novel source for crop improvement[5]. On the other hand, callus culture and plant regeneration provide a useful technique on clonal mass propagation in F_1 hybrids and transformants. In this case, somaclonal variations seem to be a serious problem. For both crop improvement and mass propagation, it is necessary to analyze and control the occurrence of somaclonal variations.

There are a number of reports describing the influence of plant growth regulators in the callus culture medium and of the duration of culture phase on cytological and genetic instability[6,7]. In addition, such variations were affected by the mode of regeneration; for example, regeneration from protoplast versus leaf explant[8], and regeneration via somatic embryogenesis versus organogenesis[9]. However, at this time, there have been no studies conducted to investigate the regeneration condition with different *in vitro* factors. It would be difficult to estimate the effect of regeneration conditions on somaclonal variation because, in general, changes in composition and concentration of the regeneration medium significantly alters regeneration frequency.

In our previous study, we had developed two kinds of regeneration conditions, namely a static culture on a semi-solid medium and a suspension culture in a liquid medium, using a suspension of cultured rice cells[9,10]. Under these conditions, almost the same number of plants were regenerated from the same volume of cells (*ca.* 50 plantlets/20 mg cell clusters). Using these methods, we compared the regeneration frequency of green and albino plants between the liquid and the semi-solid regeneration media[11]. We found that only albino but not green plants were reduced in the liquid regeneration medium compared to the semi-solid medium. In the present study, the comparisons of somaclonal variation in heading date, culm length and seed fertility were made using these two regeneration conditions.

Calli were initiated from mature seeds of rice (*Oryza sativa* L. cv. Sasanishiki). Induction of callus and initiation of suspension culture were as described by Tsukahara and Hirosawa[9,12]. Six-week-subcultured cells were used in this study for regeneration. Plant regeneration on the semi-solid medium and in the liquid medium were as described by Tsukahara and Hirosawa[9] and Tsukahara *et al.*[10], respectively. In brief, approximately 20 mg of cell clusters were transferred to 9 cm plastic plates containing MS[13] semi-solid medium supplemented with 30 g/l sucrose, 30 g/l

sorbitol, 2 g/l casein, acid hydrolysate (CH), 5 mM 2-(N-morpholino) ethanesulfonic acid (MES) (pH 5.8), 2 mg/l 1-Naphthaleneacetic acid (NAA), 1 mg/l kinetin and 4 g/l gellan gum and to 300-ml flasks containing N6[14] liquid medium supplemented with 10 g/l sucrose, 30 g/l sorbitol, 0.1 g/l CH, 12 mM proline, 5 mM MES (pH 5.8), 0.1 m/l NAA and 0.1 mg/l kinetin. After 6 weeks, the regenerated plantlets with 5-20 mm green shoots were transferred to MS medium supplemented with 30 g/l sucrose and 3 g/l gellan gum. After 4 weeks, plants of 8-15 cm in height were transplanted into soil and grown in a greenhouse. The control was Sasanishiki rice seeds that were sown using a similar method to that of regenerated plants. Seventy-five plantlets of each plant line were transferred to the paddy field. They were planted individually in three rows with a row space of 30 cm and a hill space of 18 cm. Fertilizer and chemicals were applied according to the standard paddy field culture methods.

Twenty-three plants in the center row except two plants at both ends were used for data collection. Heading date was determined on the day that the first panicle was observed growing on each plant. Culm length and seed fertility were measured using the longest stem in each plant. Analysis of variance was performed and Tukey's Multiple Range Test (TMRT) was used to compare the agronomic characters.

Average heading date results of plants regenerated on the semi-solid medium was earlier than that of plants from the liquid medium, and both plant lines also showed earlier heading date than control (**Table 1**). However, all plants regenerated from the semi-solid medium were variants in heading date. More than half of the regenerated plants from the liquid medium were in the same range as control (**Table 2**). None of the regenerated plants showed a later heading date than Aug. 5 th. The average culm length was shorter for regenerated plants than that of the control. Also, there were no significant differences observed between the two regeneration methods (**Table 1**). However, the proportion of short culm variants were more frequent in plants regenerated on the semi-solid medium than in the liquid medium (**Table 2**). Average seed fertility of plants regenerat-

Table 1. Mean for agronomic characters among control plants and regenerated plants.

Line	Heading date	Culm length (cm)	Seed fertility (%)
Control*1	Aug. 2±2 A	86.0±9.0 A	94.0± 2.6 A
Solid*2	Jul. 24±2 B	65.3±6.4 B	53.1±33.2 B
Liquid*3	Jul. 30±3 C	71.6±8.3 B	69.2±31.7 C

*1 Control plants from seeds.
*2 Plants regenerated on the semi-solid medium.
*3 Plants regenerated in the liquid medium.
In each column, numbers follwed by a common letter are not significantly differrent at 5% level by TMRT.

Table 2. Number of variants in agronomic characters among control plants and regenerated plants.

Line	Heading date		Culm length		Seed fertility	
	Jul. 30-Aug. 5	Others	≧75 cm	<75 cm	≧78%	<78%
Control*1	23	0	23	0	23	0
Solid*2	0	23	2	21	8	15
Liquid*3	15	8	13	10	15	8

*1 Control plants from seeds.
*2 Plants regenerated on the semi-solid medium.
*3 Plants regenerated in the liquid medium.

ed on the semi-solid medium was lower than that of plants in the liquid medium (**Table 1**). The proportion of variants that showed less than 78% of fertility was almost two times higher in plants regenerated on the semi-solid medium than the liquid medium (**Table 2**). In addition, these variant characters were also observed in the next generation.

The results of this study demonstrated that the occurrence of somaclonal variation was influenced by the regeneration condition. Both the proportion of variants and the degree of variation were smaller for regenerated plants from the liquid medium than those from the semi-solid medium. These results were consistent with our previous study on albino versus green plants[12].

There are three possible mechanisms that may explain why the regeneration condition has an effect on the proportion of variants among regenerated plants. First, somaclonal variation may occur and/or amplify during regeneration, and the differences of composition and concentration between regeneration media alters the degree of variation. We did not demonstrate the effect that the different media compositions have on somaclonal variation because, in our previous study, we have shown that the different media compositions did not affect the regeneration frequency of albino[11]. Second, somaclonal variation may recover during regeneration. It is well known that tissue culture resulted in changes of methylation status of DNA[15]. It is also reported that medium composition influenced the methylation patterns in cultured carrot roots[16] and potato tubers[17]. If somaclonal variation is due to gene inactivation by DNA hypermethylation, there is a possibility that the normal phenotypes can be recovered by hypomethylation. The third possible mechanism is that the regeneration process in the liquid medium may have stronger selection pressure on regenerated plants having somaclonal variation than the semi-solid medium. It has been shown that in regenerated plants from embryogenic calli, a strong selection in favor of plant regeneration from cytologically normal cells was observed[18]. In order to confirm the above possibilities, analysis of variation at molecular level during regeneration will be needed.

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《和文要約》

イネ農業形質の培養変異に及ぼす再分化方法の影響

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培養条件の異なる2つの再分化系，すなわち固体培地での静置培養系と液体培地を用いた懸濁培養系から得られた再分化植物において，出穂日・稈長・稔実率に対する変異の出現率を比較した．いずれの形質においても，固体培地由来の植物に比べ，液体培地では変異植物の出現頻度が減少していた．