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Genetic Variation of a Single Pollen-derived Doubled Haploid Population in Rice

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ABSTRACT

Somaclonal variation was observed in the field on doubled haploid plants derived from single pollen of a rice cultivar "Hwaseongbyeo". The variations of seven quantitative traits including plant height and one qualitative trait (pubescence) in 436 lines (R_2 generation) were analyzed. The number of lines which fell beyond the boundaries of the 95% confidence intervals of the check variety, Hwaseongbyeo was checked for each quantitative trait, and of those fertility showed the highest variation frequency (85.6%), followed by plant height (77.5%), flag leaf length (66.5%), grains per panicle (42.2%), days to heading (34.5%), panicle length (30.7%) and panicles per hill (22.7%). And the variations of quantitative traits except days to flowering appeared to move in the negative direction compared to "Hwaseongbyeo".

Variability within lines was also observed for quantitative and qualitative traits. Twenty-nine R_2 lines (7%) segregated for pubescence and 130 R_2 lines (30%) showed variation with regard to fertility. This suggests that mutations usually occur before diploidization.

Twenty-nine R_2 lines representing a wide spectrum of variation were chosen for RAPD analysis. The number of lines showing DNA polymorphism compared to Hwaseongbyeo ranged 0 from to 10 according to the primer used and this seems to indicate that specific loci have highly mutable genomic site.

Key words : doubled haploid population, pollen culture, single pollen, somaclonal variation, rice, RAPDs.

Somaclonal variation induced during the culture of plant cells may provide a useful source of genetic variability for crop improvement and is well documented in many plant species. In rice, somaclonal variation has been previously described in plants regenerated from callus cultures (Oono, 1978 ; Sun et al., 1983) and from protoplast cultures (Ogura et al., 1989) and included differences in both quantitative and qualitative traits including plant height, days to heading, and sterility.

RFLP (restriction fragment length polymorphism) analysis revealed substantial variation in rice plants regenerated from non-embryogenic tissue cultures (Muller et al., 1990). Brown et al. (1990) also reported that significant levels of DNA variation were produced as a result of tissue culture and DNA variation was not significantly different between callus- and protoplast-derived rice plants.

RAPDs (randomly amplified polymorphic DNAs) technique was also employed for analyzing the genetic stability of tissue-cultured plants (Valles et al., 1993). Taylor et al. (1995) reported that RAPD analysis was suitable for detecting somaclonal variation in sugarcane subjected to prolonged tissue culture.

Regenerated plants from pollen culture are well suited as source materials for the study of somaclonal variation because of easy detection of mutated traits and homozygosity in the subsequent generations and single pollen culture is recommended to exclude the problem caused by the intrinsic explant variability.

The objectives of this study were to i) determine the relative frequency of phenotypic variation; and ii) monitor DNA polymorphism using RAPDs analysis in plants regenerated from single pollen culture.

Abbreviations for regenerated plants derived directly from pollen culture and their following generations have been referred to as R_0, R_1, R_2 in this study.

MATERIALS AND METHODS

"Hwaseongbyeo", an anther-derived *japonica* cultivar was used in this study. A vacuum anther-collecting apparatus was used to collect anthers from panicles aseptically (Moon et al., 1994). For pollen isolation and culture, procedures reported by Cho & Zapata (1990) were used with modification of media. Preculture medium was modified B5 (B5+0.5 ppm BAP+2 ppm 2,4-D+0.5 ppm IAA+5 gl^{-1} glucose+20 gl^{-1} sucrose) and callus induction medium was modified N6 medium (N6+1 mgl^{-1} 2, 4-D+0.2 ppm NAA+1 mM glutamine+3 mll^{-1} coconut water+30 gl^{-1} sucrose). For establishment of callus clones of single pollen, two week-old culture was poured on No. 1 Whatman filter papers, and then microcalli were selected under microscope. These microcalli were multiplied separately on solid callus growth medium (N6+2 ppm 2,4-D+5 gl^{-1} casein hydrolysate+0.8% agar). Among surviving clones, three fast-growing, embryogenic clones were selected and multiplied for seven months through regular subcultures. Plants were regenerated from RM3-2 medium (MS+4 ppm Kinetin +1 ppm NAA+40 ppm adenine sulfate+1.6% agar) through 20 days' subcultures.

Regenerated plants (R_0 generation) were grown in the

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glass house. Haploid and sterile plants were discarded and seed harvesting were made at fertile doubled haploid plants. R_1 generation was grown in the paddy field but progeny testing was not performed because the number of plants was not sufficient due to poor seed set on R_0 plants. R_2 generation harvested in bulk from R_1 plants per line was planted in a single row with 30 plants in the summer of 1993. In 1993 rice plants were damaged during the reproductive stage due to unusual low temperatures and this might have affectecd the measurements of traits, especially in fertility.

The middle ten plants were used for data collection. Heading date, culm length, panicle length, panicles per plant, spikelets per panicle, fertility, flag leaf length were investigated and statistically analyzed.

Twenty-nine R_2 lines showing no-within-line-variation were selected for RAPDs analysis. DNA was extracted from ten to fifteen plants per line. Twenty-five Operon primers were used. The 25 μl of PCR reaction consisted of 20 ng sample DNA, 10 mM tris-HCl (pH 8.3), 50mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.2 mM of each dNTP, 25 nM of each 10-mer primer, and 0.5U Taq polymerase. The Perkin Elmer Cetus Thermocycler model 480 was programmed for 45 cycles of 94℃ (1 min.) 35℃ (1 min.), and 72℃ (2 min.). This was followed by a final cycle of 72℃ for 5 min. PCR products were resolved by electrophoresis in a 2% gel. Two independent runs were performed for each primer and the bands not consistent over the two reactions were not counted.

RESULTS AND DISCUSSION

Plant regeneration from single-pollen derived callus

A total of 2,235 plants was regenerated from three multiplied callus clones and grown in the glass house. Their ploidy level was determined and 436 diploid plants comprising approximately 17% were selected based on phenotype.

Considerable variation in morphological traits was observed among R_0 somaclones compared to Hwaseongbyeo. R_0 plants generally had reduced height, fewer panicles, narrower leaves and lower seed set. This result is not consistent with the previous finding (Fukui, 1983) that the regenerants derived directly from tissue culture show little observable variation. This might be partly due to the expression of recessive traits at a homozygous condition because spontaneous doubling of pollen culture derived haploids were preceded by mutations. The likelihood that this phenotypic variation might be related with differences in chromosome number might be excluded because diploid plants were selected for further analysis.

Somaclonal variation of R_2 population

Substantial differences between R_2 regenerants and the control were found for all traits examined and change in pubescence of leaves was also detected (Table 1). Compared with the control, mean performance of R_2 population was inferior and moved in the negative direction ; reduced plant height and panicle length, fewer panicles and spikelets, lower fertility, and shortened flag leaf.

The number of R_2 lines which fell beyond the boundaries of 95% confidence intervals of the check was checked for each trait (Table 1). Grain fertility showed the highest variation (85.6%), followed by culm length (77.5%). Oono (1978) reported that 72% of rice plants regenerated from callus cultures showed morphological mutations. A similar level of morphological variation was also reported by Sun et al. (1983) who found that 75.8% of 950 T2 lines showed variation at least in one out of six traits measured. The finding of this study that lines normal in fertility comprised only 14.4% is not quite surprising considering that measurements of variation are prone to error and enviromental conditions affected the stability of these traits.

Another possible reason for high level of variation is

Table 1. Mean (±S.E.) and range of agronomic traits of 436 R_2 lines. A subset of 29 lines representing a wide spectrum of variation were chosen for RAPD analysis.

Trait†	Mean (±S.E.)	Range	Varied lines‡	
			No.	%
DH	122± 3.5	97~135 (120)§	152	35
CL	67± 8.6	26~ 85 (80)	338	78
PL	18± 2.6	9~ 25 (20)	134	31
PN	13± 3.5	3~ 24 (17)	99	23
SN	101±26.3	53~181 (136)	184	42
FE	64±25.9	0~ 97 (93)	373	86
FL	26± 3.9	13~ 35 (33)	290	67
PB		*Gl, gl* (*Gl*)	128	29

† DH : Days to heading, CL : Culm length, PL : Panicle length, PN : Panicles per hill, SN : Spikelets per panicle, FE : Fertility, FL : Flag leaf length, PB : Pubescence

‡ Line whose mean is outside the 95% confidence intervals of Hwaseongbyeo.

§ Mean of Hwaseongbyeo in parenthesis.

Table 2. Number of R_2 lines showing variation within the same R_2 line per trait.

Trait	No. of lines	Percentage†
	(no.)	(%)
CL	110	25
PL	42	10
PN	66	15
SN	118	27
FE	130	30
FL	38	9
PB	29	7
Total	136	31

† No. of lines divided by a total of 436 lines.

Table 3. Distribution of primers based on how many R_2 lines showed DNA polymorphisms compared with the original variety using that primer.

No. of polymorphic R_2 lines	Operon primer	
	No.	Designation
0	2	OPD-01, OPD-08
1~3	13	OPA-10, OPA-16, OPC-18, OPD-05, OPP-14, OPP-15, OPP-16, OPP-19, OPP-20, OPQ-03, OPS-20, OPW-09, OPAA-10
4~6	6	OPC-11, OPE-04, OPP-11, OPP-13, OPP-17, OPU-09
7~9	3	OPD-02, OPP-12, OPS-06
10 and more	1	OPD-09
Total	25	

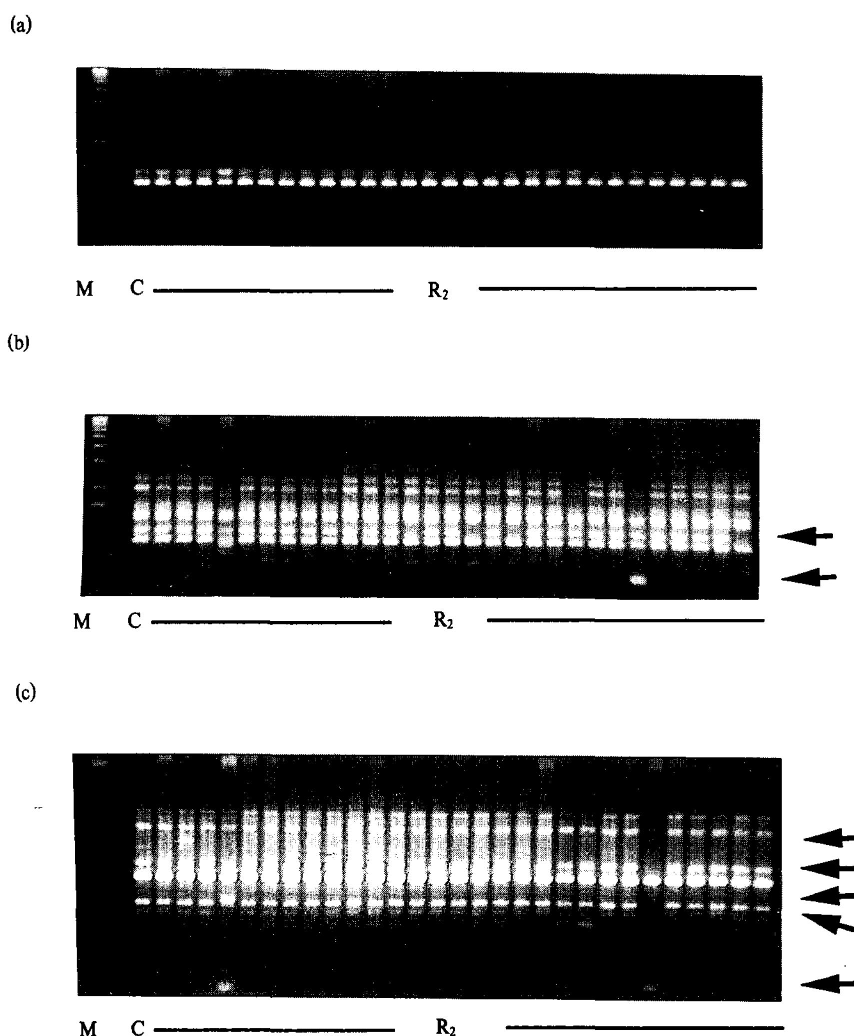

Fig. 1. Bands produced by three 10-bp Operon primers on a subset of twenty-nine R_2 lines chosen from 436 lines. Primers were (a) OPD-01, (b) OPD-02, and (c) OPU-09 (Key: M=1-kb ladder, C:Hwaseongbyeo, R_2 : 29 R_2 lines). Bands showing polymorphism between R2 lines and Hwaseongbyeo are shown by the arrows on the right.

that regenerants derived from callus cultures were maintained for a long period (7 months). Muller et al. (1990) also reported that the amount and degree of variation are greatly increased in plants derived from callus cultures maintained for a long period.

It is possible that the number of lines showing vari-

ation in this study might be a underestimation considering that somaclonal variation of a more subtle or quantitative nature is present (Larkin & Scowcroft, 1981).

Variation within lines

Doubled haploid plants are generally homozygous and the progenies from an R_0 plant should be uniform unless any mutation or gene influx happened. Variations within line (R_2) from regenerated plants derived from long-term cultured callus of haploid cells are often observed with a high frequency which cannot be explained by mutations alone during the generation advancement.

Mutations might occur before or after spontaneous doubling of pollen culture derived haploids during the culture period. If mutation precedes doubling of haploid genome, regenerated plant will be homozygous for the mutated trait and will produce homozygous progenies. However, if mutations occur after doubling of haploid genome, regenerants will be heterozygous for the trait mutated and will produce heterozygous progenies.

A large number of R_2 lines showing variation within each of lines was observed in this study. Number of lines showing variation within line is shown in Table 2. Twenty-nine R_2 lines and 130 out of 436 lines showed variation within each of the lines with respect to pubescence and fertility, respectively. Within line variation was more conspicuous in quantitative traits than in qualitative trait (pubescence) (Table 2).

RAPD analysis of R_2 lines

Twenty-nine R_2 lines showing variation in traits such as plant height, plant type, tillering ability, panicle shape, and so on were selected for RAPDs analysis. RAPDs analysis resolved 80 scorable bands in Hwaseongbyeo from twenty-five Operon primers screened (Table 3). Primers produced between 2 and 6 amplification products.

The number of R_2 lines showing DNA polymorphism compared to Hwaseongbyeo according to primers was different ranging from 0 to 10 (Table 3). PCR products amplified in 29 R_2 lines by two primers (OPD-01, OPD-08) were identical to those of Hwaseongbyeo, and it is thought that DNA polymorphism (base substitutions, short deletions or insertions) did not occur within the binding sites of those two primers. The other twenty-three primers revealed DNA polymorphism at least in one R_2 line compared to Hwaseongbyeo and the number of R_2 lines ranged from one to ten according to the primer used (Table 3, Fig. 1).

With the data obtained from this analysis it is difficult to find out a definite answer for this question why the number of lines varied according to the primer used, but the failure of two primers (OPD-01, OPD-08) to reveal the DNA polymorphism seemed to indicate that some primers span a highly mutable genomic site. This is consistent with the previous finding of Taylor et al. (1995). Screening of regenerated lines with more primers and other marker systems will be necessary to test this possibility.

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