

Variation in Spikelet-Related Traits of Rice Plants Regenerated from Mature Seed-Derived Callus Culture

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Abstract : Callus is an excellent source for *in vitro* plant regeneration, but plants regenerated from callus sometimes show phenotypic and genotypic variation from the initial plants. In this study, the variation in spikelet-related traits of the rice plants regenerated from calluses and their performance in the paddy field were examined. The phenotypic variation in spikelet-related traits of the regenerated plants was not always in a reduction in their mean value. For instance, panicle length, spikelet number and fertile spikelet number of Indonesian rice genotypes Ciapus and BP-140 in the regenerated plants were significantly greater than those of the initial plants (developed from the seeds). The spikelet fertility of the regenerated rice plants was not significantly lower than that of the initial plants except in Ciapus and BP-140. The occurrence of somaclonal variants varied with the genotype. Ciapus and BP-140, which induce many somaclonal variants, are suggested to be valuable for genetic, breeding or functional genomic studies, while Fatmawati, which is stable, could be used for genetic transformation study.

Key words : Callus, Regenerated plants, Rice, Spikelet fertility.

The major goal of plant breeding is to improve existing cultivars and to develop new or elite cultivars. The genetic variability induced by *in vitro* culture has been exploited to serve breeding purposes due to the fact that the plants obtained from *in vitro* culture sometimes show phenotypic and genotypic variations from the initial plant. This phenomenon is called 'somaclonal variation' (Larkin and Scowcroft, 1981), which refers to the variation arising in cell cultures, regenerated plants and their progenies (Larkin and Scowcroft, 1981; Karp, 1995). The genetic basis of somaclonal variation is not yet fully understood. However, it is postulated that the events such as large-scale deletions and gross changes in chromosome structure/number and directed and undirected point mutations, transposon activation and epigenetic changes (Kaeppler et al., 2000; Jain, 2001) such as DNA methylation, hystone acetylation and chromatin remodeling (Joyce et al., 2003), contribute to the *in vitro* variations. Somaclonal variation is considered to be a useful source of variation and has been demonstrated to be valuable in rice (Zheng et al., 1989; Ling et al., 1989; Yamamoto et al., 1997). Somaclonal variants tolerant to sheath blight, *Rhizoctonia solani* (Xie et al., 1992); drought (Adkins et al., 1995); chilling (Bertin and Bouharmont, 1997); aluminium (Jan et al., 1997); salinity (Lutts et al., 2001); and blast, *Pyricularia grisea* (de Araujo and Prabhu, 2002) are successful examples of rice plants obtained by *in vitro* selection.

A wide range of altered phenotypic expression in regenerated plants can be found, such as, chlorophyll deficiency, dwarfs, seed characteristics, reproductive structures, and necrotic leaves (Phillips et al., 1994). A reduction in spikelet fertility or changes in agronomic traits has been reported to occur in *in vitro*-regenerated plants (Lee et al., 1999; Rongbai et al., 1999). However, in contrast, somaclonal variants are undesired when the tissue culture protocols applied for developing transgenic plants since the occurrence of somaclonal variants will complicate the evaluation of the effects of inserted gene(s), thus constitutes a major problems (Lutts et al., 2001). Jiang et al. (2000) reported that particle bombardment using callus as a target tissue produced 8% aberrant phenotypes among the transgenic plants. To develop stable transgenic rice plants expressing the genes of interest while retaining the genomic and phenotypic integrity, a preliminary test on the possibility of somaclonal variants of callus-regenerated plants among rice genotypes is therefore considered necessary. The present paper was designed to evaluate the spikelet-related traits of R₀ plants in comparison with those of the initial genotypes and to evaluate the performance of regenerated plants in the field. Spikelet fertility and its related traits are the main concern of *in vitro* regenerated plants especially for rice in which reproductive parts (i.e. grains) are harvested for many valuable uses.

Table 1. Comparative performance of the plants regenerated from callus and the initial genotypes grown in a glasshouse.

| Genotype | Origin | Number of plants | Plant height (cm) | Panicle length (cm) | Spikelet fertility (%) | Ratio of filled to unfilled spikelets | Spikelet number | Fertile spikelet number | 100-grain weight (g) ¹⁾ |
|------------|--------|------------------|--------------------------|--------------------------|--------------------------|---------------------------------------|-------------------------|--------------------------|------------------------------------|
| Fatmawati | Seed | 21 | 94.4 ± 1.4 | 26.7 ± 0.5 | 44.3 ± 3.3 | 0.92 ± 0.1 | 154 ± 14.9 | 73.6 ± 8.9 | 2.88 ± 0.05 |
| | Callus | 166 | 92.6 ± 0.8 ^{ns} | 25.9 ± 0.2 ^{ns} | 44.8 ± 1.6 ^{ns} | 1.13 ± 0.1 ^{ns} | 149 ± 4.2 ^{ns} | 64.6 ± 2.8 ^{ns} | 2.90 ± 0.04 ^{ns} |
| Ciapus | Seed | 36 | 87.4 ± 1.3 | 20.1 ± 0.6 | 75.6 ± 2.3 | 5.61 ± 1.5 | 80 ± 5.5 | 58.6 ± 3.8 | 3.15 ± 0.03 |
| | Callus | 122 | 89.1 ± 6.5 ^{ns} | 21.9 ± 0.2 ^{**} | 67.9 ± 1.1 ^{**} | 2.80 ± 1.2 ^{ns} | 102 ± 2.5 ^{**} | 69.1 ± 1.8 ^{**} | 3.23 ± 0.02 [*] |
| BP-23 | Seed | 41 | 83.7 ± 0.7 | 20.8 ± 0.4 | 80.3 ± 2.8 | 11.21 ± 2.0 | 68 ± 3.6 | 52.3 ± 2.7 | 2.71 ± 0.02 |
| | Callus | 36 | 88.0 ± 1.2 ^{**} | 23.7 ± 0.4 ^{**} | 75.3 ± 3.3 ^{ns} | 4.13 ± 0.5 ^{**} | 92 ± 4.1 ^{**} | 67.8 ± 3.9 ^{**} | 2.63 ± 0.03 [*] |
| BP-140 | Seed | 32 | 94.9 ± 0.9 | 23.7 ± 0.7 | 65.4 ± 3.2 | 3.53 ± 0.8 | 106 ± 8.7 | 64.2 ± 6.1 | 3.55 ± 0.02 |
| | Callus | 157 | 90.3 ± 0.7 ^{**} | 23.8 ± 0.2 ^{ns} | 47.0 ± 1.1 ^{**} | 1.03 ± 0.1 ^{**} | 116 ± 2.3 ^{ns} | 55.6 ± 1.8 ^{ns} | 3.62 ± 0.03 [*] |
| BP-360-3 | Seed | 29 | 90.3 ± 1.5 | 23.5 ± 0.5 | 59.4 ± 3.8 | 2.78 ± 0.7 | 109 ± 8.0 | 65.3 ± 6.6 | 2.93 ± 0.01 |
| | Callus | 42 | 96.8 ± 1.7 ^{**} | 23.9 ± 0.5 ^{ns} | 53.8 ± 2.3 ^{ns} | 1.40 ± 0.1 ^{ns} | 129 ± 6.0 [*] | 71.3 ± 5.2 ^{ns} | 2.86 ± 0.02 ^{**} |
| Nipponbare | Seed | 31 | 83.6 ± 1.1 | 16.3 ± 0.5 | 92.1 ± 1.7 | 25.24 ± 3.3 | 68 ± 3.3 | 62.8 ± 3.4 | 2.84 ± 0.02 |
| | Callus | 19 | 79.9 ± 1.8 ^{ns} | 16.7 ± 0.3 ^{ns} | 92.2 ± 0.9 ^{ns} | 17.38 ± 2.9 ^{ns} | 70 ± 4.3 ^{ns} | 64.8 ± 4.1 ^{ns} | 2.74 ± 0.01 ^{**} |

Differences between mean values of plants regenerated from callus and those of initial plants of the corresponding genotype are non-significant (ns) or significant at $P = 0.05$ (*) or $P = 0.01$ (**) by a Student *t*-test with considering homogeneity of variance by Levene's test. Data show mean values with standard error of means.

¹⁾ Data were taken from 15 samples of each genotype at a water content of 15%.

Materials and Methods

Five Indonesian rice genotypes Fatmawati, Ciapus, BP-23, BP-140, BP-360-3 (all *indica* ssp), and one *japonica* Nipponbare were regenerated from calluses following the methods reported previously (Carsono and Yoshida, 2006a, b). Briefly, mature seeds were cultured on callus induction medium (4 weeks), calluses were then subcultured (3 weeks) and regenerated (3-5 weeks). After acclimatization for 3-4 weeks, 542 regenerated plants randomly selected were transplanted to the pot (17.2 cm in diameter, 19.2 cm in high) containing Andosol soil with 5 to 7 seedlings per pot and grown in a glasshouse. Another 308 regenerated plants were grown in the paddy field. The initial genotypes, developed from the seeds, were also grown in the glasshouse.

At the time of sowing, pots were fertilized with equal to 60 kg N, 60 kg P₂O₅ and 60 kg K₂O ha⁻¹. Additional top-dressing with the same dosage was applied at panicle initiation. In the paddy field experiment, planting density was 15 × 30 cm spacing and 16 kg N, 16 kg P₂O₅ and 16 kg K₂O ha⁻¹ plus 16 kg ha⁻¹ of organic fertilizer was applied. The experiment was conducted at 113.26 m above sea level. In the glasshouse, plants were irrigated at 2~3-d intervals for maintaining the water availability by direct watering to the pots, while in the paddy field, water was maintained approximately 5 to 10 cm from the soil surface. The daytime temperature during the experiment ranged from 25°C to 38°C in a glasshouse and from 20°C to 33°C in the field, and the nighttime temperature from 23°C to 25°C in the glasshouse and 15°C to 25°C in the field.

Phenotypic traits were then recorded for plant

height (from ground level to tip of the tallest panicle), spikelet fertility (percentage of filled and half-filled seeds per total spikelets), panicle length, ratio of filled or half-filled spikelets to unfilled spikelets, spikelet number per panicle, fertile spikelet number per panicle, and 100-grain weight. Spikelets were characterized as unfilled when the palea and lemma folded easily when pressed with fingers (IRRI, 2002). Tillers were excluded from the analysis. Panicles were carefully harvested by hand to prevent the loss of spikelets and they were removed manually and subsequently divided into groups of filled and unfilled spikelets. Measured values of the regenerated plants were compared with those of the initial genotypes by Student *t*-test with taking the homogeneity of variance into account using Levene's test (SPSS Inc.). Analysis of variance (anova) was performed for the regenerated plants grown in the paddy field. To achieve a normal distribution, data were transformed; however, transformation did not result in marked improvement of the distribution. Therefore, nontransformed data were used throughout (Steel and Torrie, 1980).

Results and Discussion

1. Comparison of spikelet-related traits of regenerated plants grown in the glasshouse with those of the initial plants

Spikelet-related traits of rice plants regenerated from mature seed-derived callus culture are important to be explored since it is a crucial determinant of seed set, which is a basis for both transmission to the next generation and a yield per se. In this study, 542 regenerated plants grown in a glasshouse were

Table 2. Analysis of variance for the regenerated plants grown in the paddy field.

| Source of variation | df ₁ | df ₂ | Plant height (cm) | Panicle length (cm) | Spikelet fertility (%) | Ratio of filled to unfilled spikelets | Spikelet number | Fertile spikelet number | 100-grain weight (g) ¹⁾ |
|---------------------|-----------------|-----------------|-------------------|---------------------|------------------------|---------------------------------------|-----------------|-------------------------|------------------------------------|
| Genotype | 5 | 5 | 1324.4** | 501.6** | 8486.5** | 1884.98** | 312559** | 116771** | 0.819** |
| Error | 234 | 84 | 55.8 | 3.4 | 178.6 | 58.12 | 2879 | 1375 | 0.003 |
| Total | 239 | 89 | | | | | | | |

Data show mean square value. **: significant at 0.01 probability level. df₁ and df₂: degree of freedom for the first six traits and 100-grain weight, respectively.

¹⁾Data were taken from 15 samples of each genotype at a water content of 15%.

scored for plant height and spikelet-related traits (Table 1). The spikelet fertility of callus-regenerated plants varied with the genotype ranging from 44.8% (partially fertile) in Fatmawati to 92.2% (fertile) in Nipponbare (Table 1). Low spikelet fertility observed in Fatmawati and BP-360-3 is possibly due to a high air temperature (above 35°C) which frequently observed in a glasshouse during flowering stage in summer (mid-August 2005) and most likely these genotypes are two of the heat-sensitive ones as previously reported by Satake and Yoshida (1978) and more recently by Prasad et al. (2006).

The mean spikelet fertility of callus-regenerated plants was significantly lower ($P = 0.01$) than that of the initial plants in Ciapus and BP-140, but not in other genotypes (Table 1). This particular trait in the regenerated plants tended to be lower than that in the initial plants. Lee et al. (1999) and Rongbai et al. (1999) reported a decrease of spikelet fertility in *in vitro*-regenerated plants. Thus, it is suggested that the genetic instability of callus culture of Ciapus and BP-140 in regenerating fertile plants is greater than that of the other four genotypes since the occurrence of somaclonal variation is strictly affected by genotype (Karp, 1995; Lutts et al., 1998; Joyce et al., 2003), cultivated explants (Lutts et al., 2001), medium, duration of culture (Jain, 2001) and the survival of plantlets transplanted into soil (Rongbai et al., 1999). Genetic instability of callus culture occurs due to the cells face traumatic experiences from isolation, proliferation, and may reprogram during plant regeneration which are different from natural condition. Such reprogramming or restructuring of events can create genetic and epigenetic changes resulting in a wide range of phenotypic expression (Phillips et al., 1994; Jain, 2001) as shown in the significant difference in spikelet fertility of Ciapus and BP-140. Such a phenomenon was also observed in plant height in BP-23, BP-140, and BP-360-3; panicle length in Ciapus and BP-23; ratio of filled to unfilled spikelets in BP-23 and BP-140; spikelet number in Ciapus, BP-23 and BP-360-3; fertile spikelet number in Ciapus and BP-23, and in 100-grain weight in five

genotypes except Fatmawati (Table 1). These data suggest that phenotypic alteration in the primary callus-regenerated plants is not always in a negative direction (reduction in their mean value). Possible reasons for these differences can be attributed to the genotype with regard to its sensitivity to generate genetic alteration during *in vitro* culture in the nucleus or chromosome (from a point mutation, large scale deletion, aneuploidy and or polyploidy) (Brown et al., 1990; Chatterjee and Das Gupta, 1997), in the cytoplasmic genome such as chloroplast deletion (Abe et al., 2002) or activation of transposable elements such as retrotransposons which are activated as tissue culture get older (Hirochika et al., 1996) or epigenetic mechanism such as DNA methylation event (Brown et al., 1990; Kaeppler et al., 2000). The latter even can enhance quantitative traits because several genes can be affected simultaneously (Jain, 2001), as found in this study. Moreover, it seems likely that no single event or factor controls an altered phenotype obtained from *in vitro* culture (Jain, 2001). The genotypes found to induce somaclonal variants in the present study should be then further investigated for genetic, breeding or functional genomic studies. On the other hand, genotypes that are relatively stable would be valuable for genetic transformation studies.

Judging from the significance of *t*-test on given traits in Table 1, the genetic instability of callus culture among the six genotypes was the highest in BP-23 followed by Ciapus, BP-140, BP-360-3, Nipponbare and Fatmawati in this order. Table 1 also shows that all phenotypic traits of the regenerated plants were not significantly different from those of the initial plants in Fatmawati. This suggests that Fatmawati has a high genetic stability in regenerating normal plants from callus, although for other traits that were not evaluated, variations are still possible to be found, because callus culture acts as a mutagenic (Phillips et al., 1994; Jain, 2001) and most likely changes in tissue culture occurs by a cellular stress-response mechanism (Phillips et al., 1994; Kaeppler et al., 2000; Joyce et al., 2003). Thompson et al. (1986) reported that callus DNA showed many discrete bands. Banerjee et al.

Table 3. Performance of the regenerated plants in spikelet-related traits grown in the paddy field.

| Genotype | Plant height (cm) | Panicle length (cm) | Spikelet fertility (%) | Ratio of filled to unfilled spikelets | Spikelet number | Fertile spikelet number | 100-grain weight (g) ¹⁾ |
|------------|-------------------|---------------------|------------------------|---------------------------------------|-----------------|-------------------------|------------------------------------|
| Fatmawati | 105.1 ± 1.8 a | 31.4 ± 0.5 a | 65.8 ± 1.8 c | 2.34 ± 0.2 b | 358 ± 13.9 a | 233 ± 9.9 a | 2.71 ± 0.01 e |
| Ciapus | 91.9 ± 0.9 c | 25.9 ± 0.3 bc | 60.8 ± 1.5 c | 1.71 ± 0.1 b | 147 ± 5.0 d | 89 ± 3.7 d | 3.10 ± 0.01 b |
| BP-23 | 89.3 ± 0.7 c | 25.6 ± 0.2 c | 61.0 ± 3.7 c | 3.12 ± 0.5 b | 157 ± 5.6 d | 91 ± 4.5 d | 2.68 ± 0.01 e |
| BP-140 | 100.5 ± 1.2 b | 26.6 ± 0.3 b | 48.8 ± 1.9 d | 1.07 ± 0.1 b | 251 ± 12.5 b | 121 ± 6.7 c | 3.27 ± 0.02 a |
| BP-360-3 | 98.6 ± 1.3 b | 26.0 ± 1.2 bc | 72.1 ± 1.9 b | 3.24 ± 0.3 b | 197 ± 4.3 c | 142 ± 4.8 b | 2.87 ± 0.02 c |
| Nipponbare | 98.0 ± 0.8 b | 20.3 ± 0.2 d | 92.1 ± 0.7 a | 18.99 ± 2.9 a | 117 ± 3.0 e | 107 ± 2.4 c | 2.80 ± 0.01 d |

Data were taken from 40 regenerated plants of each genotype grown in the field. Means followed by the same letter in the column were not significantly different according to Duncan's Multiple Range Test at 0.05 probability level. Data show mean values with standard error of means.

¹⁾Data were taken from 15 samples of each genotype at a water content of 15%.

(1997) and Chowdari et al. (1998) also reported that many DNA variations were detected in the regenerated plants thus more variations would be obtained. Further assessment on the next progeny using cytogenetic or molecular tools is needed to detect such variations at the chromosome or DNA levels.

2. Performance of regenerated plants grown in the paddy field

Primary callus-derived plants of six genotypes were sown in a paddy field, and 40 plants of each genotype were randomly sampled and examined for plant height and spikelet-related traits. Analysis of variance showed that all traits of the regenerated plants significantly varied with the genotype ($P = 0.01$) (Table 2), indicating that those genotypes varied widely in the traits evaluated. Spikelet number showed the greatest mean square value while 100-grain weight showed the lowest. Spikelet number may be greatly influenced by callus culture or it may vary widely in nature. Grain weight is thought to be quite constant due to a rigid hull whose size is genetically determined (Fabre et al., 2005). Further comparison using Duncan's Multiple Range Test showed that the regenerated plants of Fatmawati had the highest mean value in plant height, panicle length, spikelet number and fertile spikelet number (Table 3). Nipponbare was the most fertile genotype showing high spikelet fertility and high ratio of filled to unfilled spikelets, although BP-140 showed the lowest fertility (Table 3). This is not surprising since Nipponbare has been grown for a long period of time in Kanto Region in which Utsunomiya is located, and it has been well adapted to climatic condition in this region. The high fertility of Nipponbare or low fertility in BP-140, Ciapus, BP-23 and Fatmawati could be attributed to genotypic differences in source-sink relationship which plays a significant role in rice yield potential, or it could be possible that trait has been influenced by *in vitro* culture (Lee et al., 1999; Rongbai et al., 1999). BP-140 had the heaviest 100-grain weight

of the others (Table 3). From these results, we consider that Fatmawati, BP-140 and Ciapus are promising parents for creating the high-yielding variety.

In general, *in vitro* culture increased the variability of regenerated rice plants as already shown in other works (Adkins et al., 1995; Chatterjee and Das Gupta, 1997; Lutts et al., 1998). In this study, callus culture did not promote a high phenotypic variation as we anticipated, but some callus-derived plants of Indonesian genotypes were found to be promising for further studies.

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