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Detection of DNA Instability Induced from Tissue Culture and Irradiation in *Oryza sativa* L. by RAPD Analysis

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Abstract

As a consequence of tissue culture of rice, RAPD analysis was performed to determine whether extended culture periods as undifferentiated calli affected the subsequent genetic constancy, and whether any resulting DNA re-arrangements could be detected between sibling plants produced from the same callus. Somaclonal variation was induced at the initial stage of tissue culture and it increased with the length of culture maintenance. Of the 192 total bands, the number of polymorphic bands was 22 (11.5%), 33 (17.2%), and 49 (25.5%) in the callus of 1, 3, and 6 months culture, respectively. A significantly higher level of genotypic polymorphisms between regenerants from two different somaclones was also detected, although all the regenerants were derived from a single genotype. In comparison of DNA polymorphisms between regenerants from non-irradiated and from irradiated calli, a scope of variation spectrum by gamma ray irradiation was larger than that by tissue culture. Consideration must be given to this genomic variation where attempts are to be made to use desirable somaclonal variants for plant breeding purpose and in genetic engineering program.

Key words: Gamma rays, Intraclone, RAPD analysis, Rice, Somaclonal variation, Tissue culture

Introduction

In the past 20 years of research on plant tissue cultures, regenerated plants and its progenies have revealed a rich array of culture-induced genetic variants (Phillips et al. 1994; Lee et al. 1996; Chowdari et al. 1998; Yang et al. 1999), although several unstable or non-transmissible variants may have their basis in epigenetic changes rather than genetic changes. Epigenetic modifications can constitute false positive signals if one seeks mutational change conditioning in a particular phenotype (Nelson 1977; Schaeffer 1981).

This source of genetic variation was termed somaclonal variation by Larkin and Scowcroft (1981), who proposed that novel and desirable variants could be generated via a tissue culture cycle. Somaclonal variations can be distinguished by their biochemical, physiological, and genetic characteristics as well as by their morphological traits. Somaclonal variations, but not restricted to, derive from chromosomal rearrangements, single-gene mutants (mostly recessive), gene modification and deletion, and changes in karyotype and DNA methylation (Larkin and Scowcroft 1981; Muler et al. 1990).

The significance of tissue culture-variation or somaclonal variation in crop improvement depends upon establishing a genetic basis for this variation. Extensive work has been done on the nature of the genetic modifications responsible for somaclonal variation in many plant species: in papaya (Stiles et al. 1993); in sweet potato (Connolly et al. 1994; Lee et al. 1996); in banana (Damasco et al. 1996); in peach (Hashmi et al. 1997); in rice (Godwin et al. 1997); in *Phalaenopsis* (Chen et al. 1998); and in garlic (Barandiaran et al. 1999).

Although, it is possible to induce mutants by somaclonal variation, mutation frequencies are often lower in plant systems, in part, by inefficient recovery techniques, by the presence of multiple gene copies located on different chromosomes, and by cell-to-cell interactions in even the most ideal suspension culture systems (Schaeffer and Sharpe 1983). Appropriate methods for the creation of genetic variation must

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aim at a high mutagenic efficiency, namely, a maximum number of desired mutations within a given population size. Widholm (1977) reported that the frequency of carrot cell resistant to 5MT could be increased 10 to 20-fold by treating the cells with ethylmethane sulfonate (EMS) or ultraviolet light. The major usefulness of mutagenic treatment in *in vitro* culture is adding one allele at a time to an existing genotype (Nabors 1976; Widholm 1977), and allows selection of large numbers and multiplication of the desired genotype in a minimal space and short duration under disease free conditions, and offers an efficient method for the improvement of crop plants.

Polymorphisms generated by random amplified polymorphic DNA (RAPD) analysis have been used for fingerprinting (Connolly et al. 1994) and evaluating genetic relationships among cultivars (Stiles et al. 1993). RAPD analysis has advantages because no previous knowledge of the genome is required and rapid results are obtained in comparison with restricted fragment length polymorphism (RFLP) analysis (Connolly et al. 1994). Miklas et al. (1993) also demonstrated that RAPD markers might be associated to specific traits such as disease resistance. RAPD or any other polymerase chain reaction (PCR)-based analysis would also be an attractive method for the detection of somaclonal variations. Godwin et al. (1997) analyzed eight rice somaclones generated from mature seed-derived callus cultures by RAPD method, and identified all somaclonal families differed significantly from the original material, indicating genomic alterations occurred in all families. Muler et al. (1990) observed DNA polymorphisms in both rice regenerants from cultures derived from different callus induction periods as well as among sibling plants derived from a single callus by RFLP analysis.

The purposes of this study was to identify somaclonal variations depending on the length of culture maintenance in the callus phase, to conduct molecular analysis of regenerants induced from two different somaclones under the same condition, and to determine the degree of difference in the molecular variation between regenerants induced by *in vitro* gamma ray mutagenesis by the RAPD method.

Materials and Methods

Plant materials

Calli were initiated from embryos of hulled seeds of *Oryza sativa* L. *japonica* cvs. Dongjinbyeo and Donganbyeo that were sterilized in 80% ethanol for 30 seconds and subsequently in 10% sodium hypochlorite with 2-3 drops of tween 40 for 30 min. The embryos of the seeds were rinsed 3-4 times with sterile distilled water and placed on callus induction medium which was N6 basal medium (Chu et al. 1975) supplemented with 2 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), and pH was adjusted to 5.8 before autoclaving. All cultures were incubated in a growth chamber at 25°C in the dark until calli were obtained.

To study the somaclonal variation dependent on the length of culture maintenance in callus level, the calli induced from an embryo were sampled in terms of 1, 3 and 6 months. In order to compare the DNA polymorphisms between regenerants from non-irradiated and irradiated calli, the calli induced from cv. Donganbyeo embryos were divided into 5 to 10 mg fresh weight pieces after being exposed to gamma rays [50 Gy (1 rad=1.00×10^{-2} Gy) dose] emitted from ^{60}Co. The gamma irradiation was conducted in the radiation facility at the Korea Atomic Energy Research Institute. All selected calli were transferred to regeneration medium which was MS (Murashige and Skoog 1962) basal medium supplemented with 0.1 mg/L indolacetic acid (IAA), 5 mg/L kinetin at pH 5.8. All cultures were maintained in the culture room under kept at 27°C light (16 hr) / 25°C dark (8 hr) regime and subcultured at 4 week intervals.

DNA extraction for RAPD analysis

The embryo-induced calli harvested at a different period of culture time, plants regenerated from two different callus clones, and regenerants from non-irradiated and irradiated calli were used for detection of somaclonal variation.

Leaf tissue or callus (0.5-1 g fresh weight) was frozen in liquid nitrogen and ground with a mortar and pestle. The frozen powder was transferred to 15 mL extraction buffer [100 mM Tris-HCl (pH 8.0), 50 mM EDTA (pH 8.0), 500 mM NaCl and 10 mM β-mercaptoethanol] and 11 mL 10% (W/V) sodium dodecyl sulfate (SDS), and 5 mL 5 M potassium acetate was added. The solution was incubated at -20°C for 10 min with occasional shaking and centrifuged at 10,000 rpm at 4°C for 10 min. After the supernatant was filtered through miracloth, an equal volume of chloroform was added and mixed by inversion, then centrifuged at 10,000 rpm at 20°C for 10 min. The supernatant was transferred to new tube. The DNA was precipitated by the addition of 1.7 mL 3 M sodium acetate and 10 mL isoprophyl alcohol, and recovered by centrifugation at 10,000 rpm at 4°C for 10 min after incubation at -20°C for at least 30 min. The pellet was dried and washed twice in 80% ethyl alcohol, then resuspended in TE buffer (50 mM Tris, 10 mM EDTA, pH 8.0). The DNA was further purified by extracting twice with an equal volume of chloroform : isoamylalcohol (24 : 1, V/V). After precipitation by sodium acetate and isoprophyl alcohol, and centrifugation, the DNA pellet was gently rinsed twice with 80 % ethyl alcohol, dried, and resuspended in 200 μL distilled water. The DNA concentration was determined

using a spectrophotometer (WPA UV1101 Biotech Photometer) and the sample was diluted to 15 ng/μL with distilled water.

DNA amplification and electrophoresis

DNA amplification reaction was performed in a total volume of 25 μL containing 10x reaction buffer [100 mM Tris-HCl (pH 9.0), 400 mM KCl and 15 mM $MgCl_2$], 20 mM $MgCl_2$, 0.1 mM each of dATP, dCTP, dGTP and dTTP, 0.25 μmol of random decamer primer (OPE through OPH, Operon Technologies), 15 ng of genomic DNA, and 1.0 unit of *Taq* DNA polymerase (Bioneer). Amplification was performed in a PTC-100™ Programmable Thermal Controller (MJ Research, Inc.) programmed as follows: an initial denaturation temperature of 94°C for 5 min; 55 cycles each consisting of a denaturation step of 5 sec at 94°C, an annealing step of 30 sec at 36°C and an extension step 1 min at 72°C; followed by 5 min at 72°C.

The amplification products were analyzed by gel electrophoresis in 1.2% agarose containing ethidium bromide. In all cases, lamda phage DNA digested with *Eco* RⅠ and *Hind*Ⅲ was used as the size marker. The amplification products were visualized under UV light and photographed using SL-5GD-Photographic System.

Band pattern analysis

Polymorphism was scored on a presence or absence of bands in each line and data were analyzed for clustering using the NTSYSpc version 2.0. The results were converted into a similarity matrix utilizing the SIMQUAL (similarity for qualitative data) method. A similarity coefficient was used for cluster analysis following the UPGMA (unweighted pair grouping method of averages) method, which is one of the several SHAN (sequential, agglomerative, hierarchical, and nested) clustering methods that are available. The resulting clusters were represented in the form of a dendrogram.

Results and Discussion

Genetic instability by the culture length of somaclones

To determine whether the length of culture as undifferentiated callus affected DNA stability, 1-, 3-, and 6-month cultured calli induced from cv. Dongjinbyeo were examined by RAPD analysis. Comparative analysis of the amount of DNA polymorphisms revealed that there was a positive correlation between length of callus culture and changes in DNA stability. In the callus of 1 month culture, eleven primers out of 15 primers revealed significant polymorphism, and 22 polymorphic bands out of 192 total bands were detected (11.5% of the total number amplified), with a mean of 1.5 polymorphisms per primer (or 2.0 if only those primers which gave polymorphisms are included). The RAPD profile derived from short-term culture (1 month) calli was significantly different from that obtained from the uncultured parent, suggesting that somaclonal variation might be induced at the initial stage during tissue culture. On the other hand, in the 3 months culture, 33 polymorphic bands (17.2%) were detected and only 1 primer did not reveal any polymorphisms, with a mean of 2.2 polymorphisms per primer. Six months culture resulted in a large number of RAPD polymorphic bands: 49 (25.5%) in the 3 clones examined showed RAPD variations, with a mean of 3.3 polymorphisms per primer (Table 1). DNA polymorphisms obtained by using 2 primers (OPH-05 and OPH-07) are depicted in Figure 1. Polymorphisms increased with the increased length of cultural periods. Previous reports suggested that the frequency of somaclonal variation depends on genotypes (McCoy et al. 1992; Hashmi et al. 1997). These results provided evidence that somaclonal variation increased with cultural periods, which was in agreement with results obtained with rice calli (Yang et al. 1999) and regenerants (Muler et al. 1990).

Variation of intraclone

Regenerants from two different somaclones derived from cv.

Table 1. Distribution of RAPD in various somaclones depending on the length of culture maintenance.

Primer	Number of total bands	Numer of polymorphic bands[a]		
		1 month	3 months	6 months
OPE-02	20	4 (20.0)[b]	4 (20.0)	7 (35.0)
OPE-03	15	1 (6.7)	1 (6.7)	2 (13.3)
OPE-04	12	0 (0.0)	2 (16.7)	3 (25.0)
OPE-07	12	0 (0.0)	0 (0.0)	0 (0.0)
OPE-10	9	2 (22.2)	3 (33.3)	4 (44.4)
OPE-14	11	2 (18.2)	3 (27.3)	4 (36.4)
OPE-15	13	0 (0.0)	2 (15.4)	6 (46.2)
OPE-16	10	1 (10.0)	2 (20.0)	2 (20.0)
OPE-18	10	1 (10.0)	1 (10.0)	2 (20.0)
OPH-05	14	0 (0.0)	2 (14.3)	2 (14.3)
OPH-07	16	4 (25.0)	4 (25.0)	4 (25.0)
OPH-09	12	1 (8.3)	2 (16.7)	3 (25.0)
OPH-12	13	2 (15.4)	2 (15.4)	4 (30.8)
OPH-13	12	3 (25.0)	3 (25.0)	3 (25.0)
OPH-14	13	1 (7.7)	2 (15.4)	3 (23.1)
Total	192	22 (11.5)	33 (17.2)	49 (25.5)

[a]Number of polymorphic bands in 3 clones.
[b]percentage of polymorphic bands from total bands.

Dongjinbyeo were examined to determine whether DNA polymorphism could be detected between sibling plants derived from the same callus. Regenerants from clone A and clone B were 5 and 6, respectively. A significantly higher level of genotypic polymorphism between regenerants from two different somaclones was detected. The presence of PCR polymorphic bands in regenerants from one clone indicated a high level of sequence homology at that site (Williams et al. 1990). The regenerants in each clone shared a large proportion, clone A (83.2%) and clone B (84.3%), using RAPD markers, which indicated high homology among the regenerants from the same callus. On the other hand, the mean occurrences of polymorphism were similar in both clones.

It was found that 9 of the 12 primers tested generated at least one polymorphic band in clone A. Twenty seven polymorphic bands out of 161 total bands were detected using those 9 primers. About 3.0 polymorphic bands per primer were observed and only those primers that gave polymorphisms were included for analysis. If primers produced DNA polymorphic products were considered, the range of polymorphism per clone varied from 7.1-55.6%, with a mean of 3.1 polymorphic bands (Table

Table 2. Intraclonal variation of plants regenerated from cv. Dongjinbyeo calli by RAPD analysis.

Primer	Clone A		Clone B	
	No. of total bands	No. of polymorphic bands	No. of total bands	No. of polymorphic bands
OPE-02	16	3 (18.8)[a]	15	4 (26.7)
OPE-12	11	6 (54.6)	10	3 (30.0)
OPE-16	14	1 (7.1)	16	0 (0.0)
OPE-18	13	0 (0.0)	13	0 (0.0)
OPE-07	17	6 (35.3)	18	10 (55.6)
OPE-13	16	5 (31.3)	14	3 (21.4)
OPE-17	12	1 (8.3)	11	1 (9.1)
OPE-18	7	0 (0.0)	7	0 (0.0)
OPE-05	16	1 (6.3)	16	0 (0.0)
OPE-07	14	2 (14.3)	14	1 (7.1)
OPE-12	13	0 (0.0)	13	0 (0.0)
OPE-16	12	2 (16.7)	12	3 (25.0)
Total	161	27 (16.8)	159	25 (15.7)

[a]percentage of polymorphic bands from total bands.

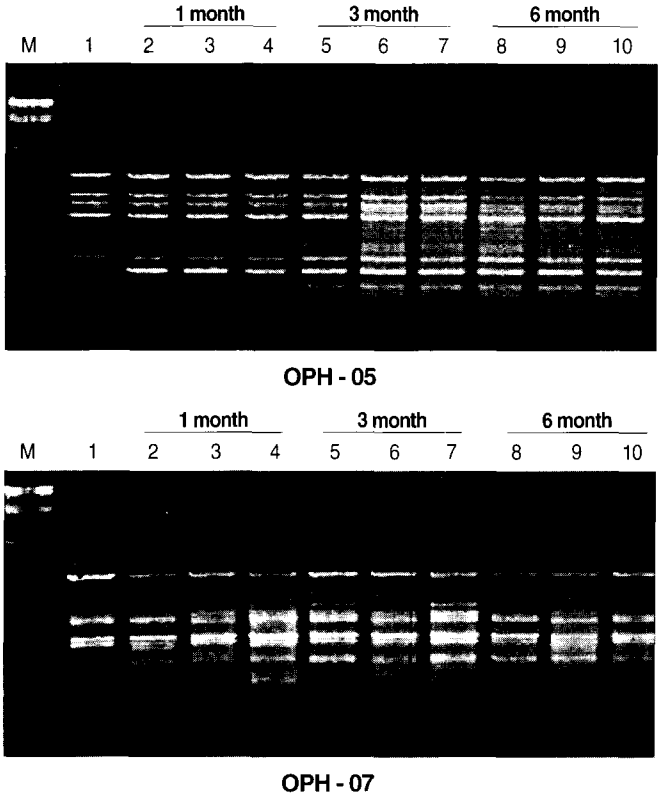

Figure 1. RAPD profiles of different lines cultured for 1 (lanes 2-4), 3 (lanes 5-7) and 6 months (lanes 8-10) obtained with primer OPH-05 and OPH-07. The RAPD profile obtained from cv. Dongjinbyeo leaf grown in greenhouse (lane 1) is shown as a control. Lane M is molecular weight marker λ digested with *Eco* RI and *Hind* III.

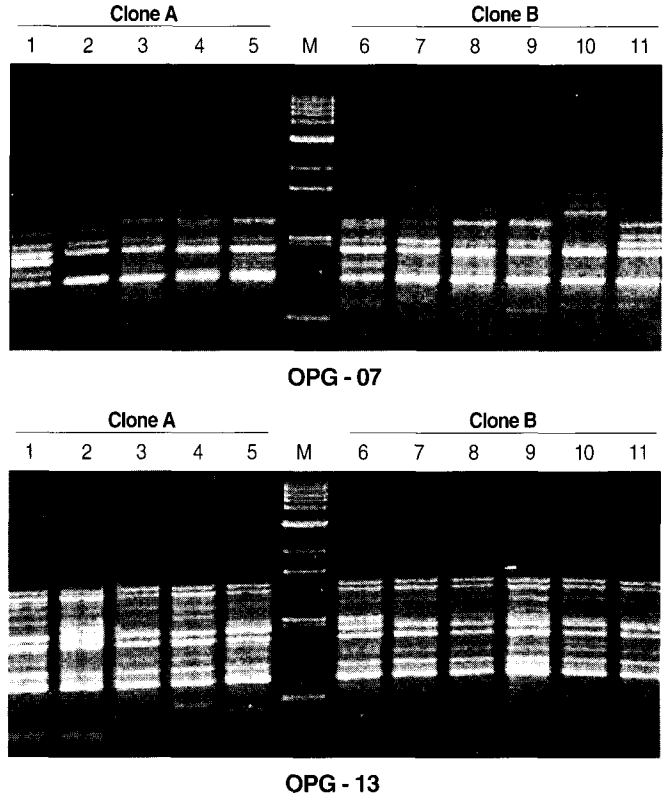

Figure 2. RAPD polymorphisms between two different somaclonal regenerants revealed after amplification with primer OPG-07 and OPG-13. M: Molecular weight marker λ digested with *Eco* RI and *Hind* III, lanes 1-5: Regenerants from clone A, lanes 6-11: Regenerants from clone B.

2). Figure 2 represents RAPD profiles between regenerants induced from two different somaclones using primer OPG-07 and OPG-13. These results suggested that genetic changes had occurred during *in vitro* culture, despite all the regenerants being derived from a single genotype. In the case where the marker was present in one genotype but not the other, there was the certainty of sequence difference. Consideration of this genomic variation must be given where attempts are to be made to use desirable somaclonal variants for plant breeding purpose and in genetic engineering programs. Rani et al. (1995) found RAPD variations among 23 micropropagated *Populus deltoides* plants originating from the same clone and morphologically similar. Bouman et al. (1992) also found intraclonal RAPD polymorphism among micropropagated *Begonia* but at a lower frequency than phenotypic variations. Our results suggested that RAPDs were useful for revealing the genetic basis for somaclonal variation. Of particular interest is pinpointing the locus of this genomic alteration. In addition, this marker will be a useful tool in determining whether the polymorphism is pre-existing, or if the genomic alteration is actually induced in culture.

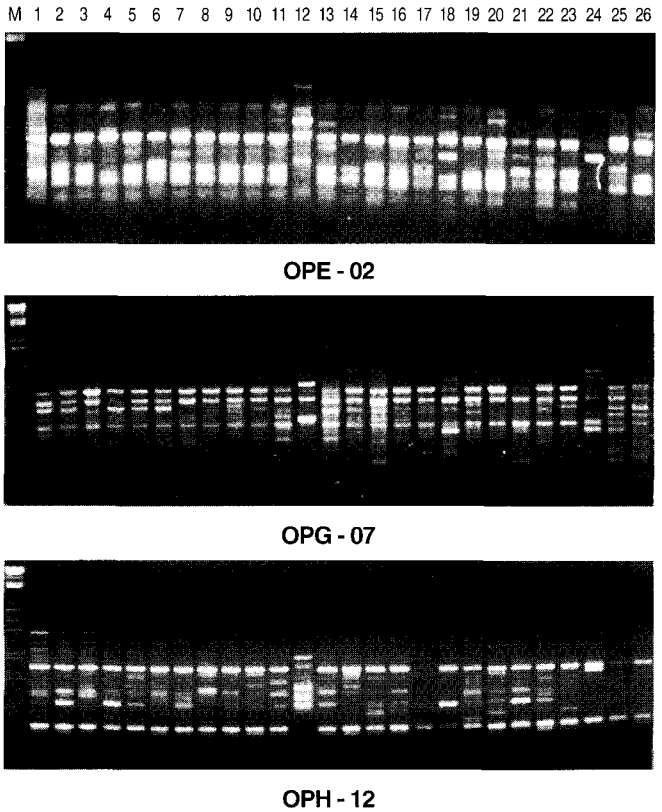

Figure 3. RAPD profiles for the detection of polymorphisms in regenerants from non-irradiated and irradiated calli in cv. Donganbyeo by using random primers, OPE-02, OPG-07, and OPH-12. M, molecular weight marker λ digested with *Eco* RI and *Hind* III; lane 1, plant from cv. Donganbyeo seed as a control; lane 2-6, regenerants from non-irradiated calli; lane 7-26, regenerants from calli irradiated with 50 Gy gamma rays.

Irradiation induced DNA variations

To inquire about whether there are the differences in the variation at molecular level between regenerants from non-irradiated and irradiated calli, RAPD profiles of one seedling generated from cv. Donganbyeo seed as a control (No. 1), five regenerants from non-irradiated calli (No. 2-6), and twenty regenerants from calli irradiated with 50 Gy gamma ray (No. 7-26) were compared. All regenerants differed significantly from the parental materials (No. 1), indicating that genomic alteration occurred in them. The number of bands for each primer varied from 13 for OPE-10 and OPH-13 to 24 for OPE-04 and OPE-14. Each primer generated a unique set of products ranging from 300 bp to 1,200 bp in size. RAPD analysis of plants regenerated from different calli exhibited DNA polymorphisms regardless of where the plants had a normal phenotype. Especially, NO. 12 and 13 revealed significant difference with RAPD profiles from the original variety and the other variants.

As shown in Figure 3, the RAPD profiles derived from 5MT resistant regenerants using primer OPE-02, OPG-07, and OPH-12 were significantly different from that obtained from the uncultured original variety Donganbyeo. The present study demonstrated the sensitivity of RAPD for somaclonal variation analysis in cultured rice cells. This conclusion is in agreement with the results that RAPDs are useful for establishing a genetic basis for somaclonal variation.

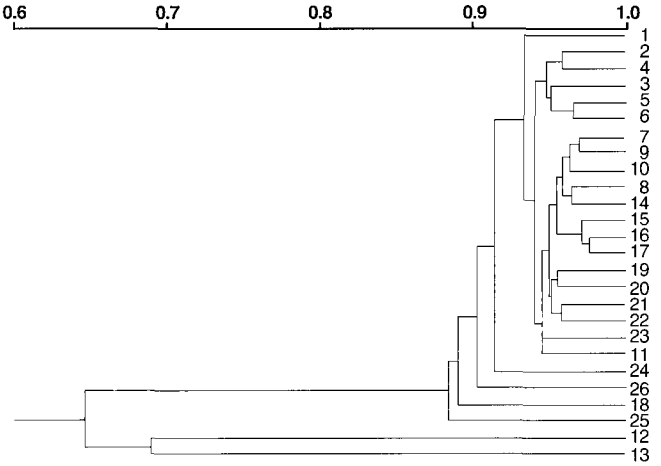

Figure 4. Dendrogram of cv. Donganbyeo variants determined from 451 RAPD markers. Similarity between variants was measured using a UN1 coefficient with clustering performed by the UPGMA strategy. No. 1, plant from control seed; No. 2-6, regenerants from non-irradiated calli; No. 7-26, regenerants from calli irradiated with 50 Gy gamma ray.

The pair-wise coefficients of correlation between control plant and regenerants from putative 5MT resistant non-irradiated and irradiated calli were clustered using the UPGMA method (Figure 4). Relationship among regenerants from non-irradiated calli was grouped to a single branch of the tree and, as expected, had a similarity coefficient above 93% with the control. In the majority of regenerants from irradiated calli, similarity coefficients with the control were above 88%, while No. 12 and No. 13 had similarity coefficient less than 66% and grouped separately. This means that the width of variation spectrum by gamma irradiation was wider than that of non-irradiation.

Most plants regenerated from calli obtained from irradiated and non-irradiated calli were phenotypically similar to those derived from seed, but RAPD profiles showed many polymorphisms among the regenerated plants from non-irradiated and irradiated calli. These results revealed the drastic genetic rearrangements that are responsible for a high frequency of genetic variation among regenerated plants (Larkin and Scowcroft 1981).

Our results clearly indicated that irradiation at callus stages increased a scope of variation spectrum. The plants from non-irradiated culture, as expected, had similarity coefficient above 93% with the control in the classification of regenerants using UPGMA method. On the other hand, the similarity coefficients of plants from irradiated culture revealed various distribution as 62.7-94.1%. This revealed a relatively high degree of genetic variability by mutagen treatment.

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