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Variations in Sweetpotato Regenerates from Gamma-ray Irradiated Embryogenic Callus

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Abstract

Radiation induced and somaclonal variations were investigated in the regenerates from gamma irradiated and controlled embryogenic callus (EC) of sweetpotato cvs., Yulmi and White Star by morphological, RAPD and AFLP analysis. Most (approx. 90%) of the EC produced somatic embryos developed into plantlets after being transferred to the auxin-free medium. The frequency of morphological variants derived from the irradiated callus ranged from 3 to 7.8% compared to 0.1-1.1% of that derived from the non-irradiated. Morphological variants were selected from the regenerates and analyzed by RAPD and AFLP procedures. RAPD polymorphisms of Yulmi and White Star regenerates from irradiated calli were 8.8% and 6.1%, respectively. However, the polymorphisms among regenerates from the non-irradiation treatment in these two cultivars were non-detectable and 3%, respectively. AFLP polymorphisms of Yulmi and White Star regenerates from irradiated calli were 29.9% and 28.6%, respectively. while the frequencies for those form non-irradiated calli were 8.5% and 5.6%, respectively. Both the control plants and variants from the non-irradiated were clustered together, while variants from irradiated were separated from the group by Nearest-Neighbor-Interchange Branch Swapping.

Abbreviation: EC (Embryogenic callus), AFLP (Amplified Fragment Length Polymorphism), RAPD (Random amplified polymorphic DNA)

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Introduction

The sweetpotato (*Ipomoea batatas* L.) is an important food crop that provides carbohydrate and protein to a large sector of the world population and also has potential as a biomass species for methane and ethanol production (Liu and Cantliffe, 1984). Despite its enormous significance in world's agriculture, studies on the breeding of this species are still insufficient. The conventional methods for breeding have some limitations because of the limited genetic base, and the self- and cross-incompatibility of this crop.

In a wide range of plant species, stable mutant traits have appeared among plants regenerated from cultured cells and tissues (Larkin and Scowcroft, 1981). Such genetic variation, termed somaclonal variation, has been considered a source of new plant genotypes for breeding (Brettell et al., 1986; Brown et al., 1993; Hadi and Bridgen, 1996). However, combination of radiation techniques with *in vitro* culture methods can speed up breeding programmes, from the generation of variability, through selection, to multiplication of the new genotypes (Maluszynski et al., 1995)

Genetic improvement by using such *in vitro* mutagenesis has been attempted in various crop plants such as pineapple (Lapade et al., 1995), banana (Matsumoto and Yamaguchi, 1991; Raop et al., 1995), and grape (Kuksova et al., 1997). This variation was caused by gross alterations in the chromosome number or structure, point mutations, mitotic recombination, deletion of DNA, transposition, or methylation of DNA sequences in nuclear, mitochondrial or chloroplast genomes (Lee and Phillips, 1988).

Several strategies can be used to assess the genetic integrity of *in vitro*-derived clones, but most of them have limitations. Karyological analysis cannot reveal alterations in specific genes or small chromosomal rearrangements. Isozyme markers have provided a convenient method for the detection of genetic changes but they are subjected to ontogenic variation, and only DNA regions coding for soluble proteins can be detected. RFLP markers are time-consuming, costly and require specific probes and large amounts of plant tissue. The PCR-based random amplified polymorphic DNA (RAPD) assay (Williams et al., 1990; Wachira et al., 1995) can overcome most limitations of RFLPs, but it is low in reproducibility because of sensitivity to experimental conditions.

The amplified fragment length polymorphism (AFLP) developed by Vos et al. (1995) is powerful and reliable in DNA fingerprinting. The reproducibility of AFLP is ensured by using restriction site-specific adapters and adapter-specific primers with a variable number of selective nucleotides under stringent amplification conditions (Vos et al., 1995). The number of polymorphisms detected per reaction is much higher than that produced by RFLPs or RAPDs because of the greater number of loci sampled in a single assay.

The aims of the work reported in this paper were: (1) to compare the frequency of embryogenic callus formation in shoot tips, leaves, and petioles of sweetpotato; (2) to determinate the effectiveness of radiation for induction of variants; (3) to test whether such mutation breeding systems might be useful in obtaining variants of this species.

Materials and Methods

Plant materials and induction of embryogenic callus (EC)

Two sweetpotato cultivars, Yulmi and White Star, were multiplied by clonal propagation. Nodal stem cuttings (10 mm) taken from 5-week-old plants were sterilized with sodium hypochlorite (5%) for 10 min, washed thoroughly with sterile distilled water and inoculated on MS basal medium containing 30 g/L sucrose, 4 g/L phytagel and adjusted to pH 5.7 before autoclaving. In order to induce embryogenic callus, shoot meristems (1 mm), leaves (5 × 5 mm) and petioles (5 mm) taken from 3-week-old *in vitro* plants, 15 explants per 9 cm petri dish, were placed on MS basal medium supplemented with 1 mg/L 2,4-D, 30 g/L sucrose, solidified with 4 g/L phytagel, adjusted to pH 5.7 before autoclaving, incubated in darkness at 27°C and subcultured at 3-week intervals.

Gamma irradiation, regeneration and evaluation

Embryogenic callus with the diameter of about 0.5-1 mm were irradiated with a ^{60}Co source. The irradiation dosages were 0, 30, 50, 70, and 90 Gy. Radiation treated and untreated calli (25 calli per petri dish) were transferred to the callus induction medium for 4 weeks.

Plantlets were regenerated on 50 mL2,4-D free MS medium in a 250 mL glass bottle and maintained in a growth chamber at 25 ± 1°C under fluorescent light for 16h/day at 700 μmol/m²/s. After 30 days *in vitro* culture, the regenerates were acclimatized for 2 weeks in a pot (55 × 45 × 12 cm) containing sand (100% humidity) and covered with transparent polyvinyl sheet and then transferred to a pot with the mixture of vermiculite and sand (1:1) (60% humidity) for 3 weeks.

Investigation of morphological variation

The field was covered with a black polyvinyl sheet after fertilizer (N-P-K) application at 3.5, 0.6, 3.5 kg/10a, and the 3 week old plants were planted on the hill of furrows (60 × 30 cm) on May 25th, 2000 for the investigation of morphological variation. Of the regenerates, 685 plants of Yulmi (260 at 0 Gy, 215 at 30 Gy, 65 at 50 Gy, 60 at 70 Gy, 85 at 90 Gy) and 915 plants of White Star (175 at 0 Gy, 330 at 30 Gy, 135 at 50 Gy, 185 at 70 Gy, 90 at 90 Gy) were evaluated for morphological variation, such as chlorophyll deficiency, stem color, leaf shape and plant type compared to the control by field observation at the full grown stage, 14 weeks after transplanting.

RAPD and AFLP analyses

DNA was extracted from the leaves of regenerates exhibiting phenotypic variation in Table 2 (14 plants of Yulmi and 16 plants of White Star) by the method of Saghai-Maroof et al. (1984). Ten 12-mer primers (Wako, Japan) were used for polymerase chain reaction (PCR) amplification. For RAPD marker PCR amplification was carried out in 25 μL volumes containing 15 ng DNA, 10 mM Tris · HCl pH 8.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (w/v) gelatin, 100 μm dNTPs, 1 U *Taq* DNA polymerase, and 0.2 μm primer. Amplifications were performed with a Perkin-Elmer 480 Thermal Cycler in the following conditions: one step 5 min predenaturation at 94°C ; 55 cycles, each consisting of a denaturation step of 5 sec at 94°C, an annealing step of 1min at 37°C, an extension step of 30 sec at 72°C and a post-extension step of 5 min at 72°C. The amplification products were electrophoresed in 1.5% (w/v) agarose gel and visualized by staining with ethidi-

um bromide. AFLP analysis was performed according to Vos et al. (1995). Restriction endenuclease *Eco*RI and *Mse*I were used.

Data analysis

RAPD and AFLP polymorphic bands were scored as present (1) or absent (0). To estimate phylogeny among all variants, heuristic search to find parsimonious unweighted tree was carried out by Nearest-Neighbor-Interchange (NNI) branch swapping (PAUP 4.0 beta version, Swofford, 1998). The chi-square test and Statistical Packages for Social Sciences (SPSSX 1983) were used to compare the various treatments.

Results

Within 3 to 4 weeks, explants of shoot tips, leaves and petioles start to became yellowish, nodular and swollen, and to form embryogenic calli on media containing 2,4-D (Figure 1A). Table 1 shows the effect of 2,4-D on EC formation from the Yulmi and White Star explants. For EC formation, 2,4-D was required. The rate of EC formation on medium containing 1 mg/L 2,4-D was higher than that in any other auxin concentration. No difference was observed between the two cultivars for EC formation frequency.

After 2 to 3 weeks of culture on 2,4-D-free medium, numerous globular somatic embryos arose from the non-irradiated and irradiated calli (Figure 1B). Most (approx. 90%) of the EC produced somatic embryos that developed into plantlets after being transferred to the auxin-free medium (Figure 1C). Approximately 93% of the acclimatized plantlets survived, except those showing extreme phenotypic variations. Figure 1D and E show morphological variants of White Star and Table 2 shows the frequencies of the variants of the regenerated plants. Morphological

Table 1. Frequency of embryogenic callus formation from those types of sweetpotato explants of two cultivars cultured on MS medium supplemented with various 2,4-D levels for 30 days.

Cultivars	2,4-D treatment (mg/L)	No. of explants induced callus		
		Shoot meristem (%)	Petiole (%)	Leaf (%)
Yulmi	0	0 (0)	0 (0)	0 (0)
	0.5	3.0±0.6 (20.0)	2.0±0.6 (13.3)	1.7±0.3 (11.3)
	1	11.0±1.5 (73.3)	5.3±0.9 (35.3)	5.0±0.6 (33.0)
	2	5.0±1.0 (33.3)	3.6±0.7 (24.0)	3.7±0.3 (23.3)
White Star	0	0 (0)	0 (0)	0 (0)
	0.5	4.0±1.0 (26.7)	2.0±0.6 (13.3)	1.0±0.6 (6.6)
	1	12.0±1.5 (80.0)	4.7±0.7 (31.3)	5.0±0.6 (33.3)
	2	4.0±0.5 (26.7)	3.0±0.6 (20.0)	2.3±0.7 (15.3)

Each value represents the mean±SE of 3 replicates. (15 explants per petri dish).

Table 2. Morphological variants observed among regenerated plants sweetpotato derived from gamma ray irradiated or control embryogenic callus.

Cultivars	Radiation dose (Gy)	No. of investigated plants	No. of variants					
			Chlorophyll deficiency	Stem Color	Leaf Type	Plant Type	Total	%
Yulmi	0	260	1	-	-	1	2	0.1
	30	215	2	-	2	3	7	3.3 a
	50	65	2	-	-	2	4	6.2 a
	70	60	1	-	-	2	3	5.0 a
	90	85	1	1	-	3	5	5.9 a
Total		685	7	1	2	11	21	3.1
White Star	0	175	1	-	1	-	2	1.1
	30	330	1	4	3	2	10	3.0 b
	50	135	2	3	2	2	9	6.7 a
	70	185	3	4	4	3	14	7.6 a
	90	90	1	3	2	1	7	7.8 a
Total		915	8	14	12	8	42	4.6

a, b: Significantly different within column at P=0.01 and 0.05 using chi-square test, respectively.

variants were chlorophyll deficient, purple stem color, leaf type (such as severe lobed, wide and narrow leaf shape), plant type (such as dwarf, multibranches and fascination type), and shortened and thick internodes, petioles, etc. Only one of the chlorophyll deficient and one of the plant type variant appeared in the non-irradiated Yulmi, while six chlorophyll deficient, 1 stem color change, 2 leaf types and 10 plant type variants were found in Yulmi irradiated with 30-90 Gy gamma ray. The frequency of the variants at the 50Gy dose was higher than the other doses.

Morphological variants in White Star increased parallel to gamma radiation dose. The frequency of the variation for the 90 Gy dose was the highest of all the doses in White Star. Of the two cultivars calli, White Star callus was more sensitive to irradiation (Table 2). Frequencies of the variants derived from irradiated callus ranged from 3% to 7.8%, compared to 0.1-1.1% in non-irradiated callus. The frequencies of variants from the irradiated callus were 2.7-77 times higher than that of non-irradiated depending on the cultivars and radiation doses.

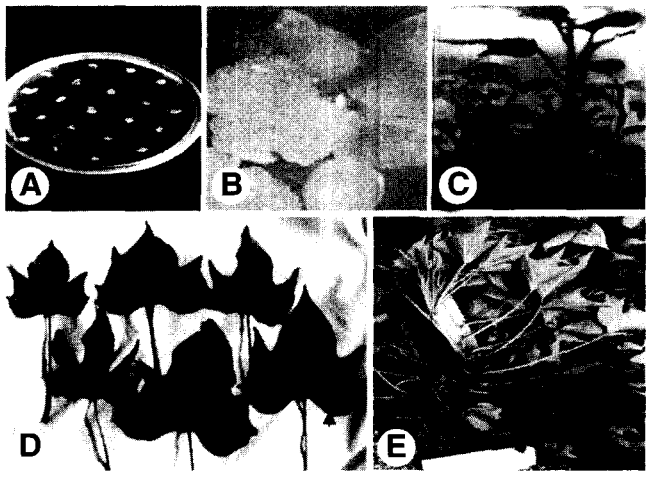

Figure 1. Plant regeneration from embryogenic calli irradiated with gamma ray in sweetpotato. A, Embryogenic calli irradiated; B, Somatic embryo formation; C, Plantlets regenerated from the calli; D, Leaf shape variants (arrow is original plant leaf); E, Fascinated stem variant of White Star observed in field.

To detect the variation of the regenerates at the DNA level, AFLP and RAPD analyses were carried out. Ten primers were selected based on their ability to produce reproducible RAPD bands with template DNA from Yulmi, White Star and the regenerates. The 10 primers used in the analysis yielded 79 bands in Yulmi and 65 bands in White Star (Table 3), respectively. The number of bands for each primer varied ranging from three for primer B-45 to 13 for B-41. Six primers (Wako B-21, -22, -31, -41, -44 and -45) out of ten revealed polymorphism in the variants compared to the original plant, but the rest (B-27, -32, -43 and -46) produced only monomorphic bands. Yulmi regenerates from gamma-treated callus showed variations of DNA levels with five primers out of ten primers used, i.e. 1 out of 9 plants for primers B21, B22 and B44 (11.1%), 2 out of 9 plants for primers B31 and B41 (22.2%). However, the regenerates from non-irradiated callus did not show any variation. White Star regenerates from the gamma-treated callus displayed variation in two primers, i. e. 3 out of 11 plants in B21 (27.3%) and 1 out of 11 plants in B41 (9.1%). Regenerates from untreated callus showed a variation for two primers i.e. 1 out of 4 plants in B21 (25%) and 1 out of 4 plants in B45 (25%). The polymorphism rate of the Yulmi regenerates from gamma-treated

Table 3. The oligonucleotide primers selected for the RAPD analysis and polymorphic data *of non-irradiated and irradiated variants in Yulmi and White Star.

Wako primers	No. of amplified bands		P/T			
			Yulmi		White Star	
	Yulmi	White Star	A	B	A	B
B-21	7	7	0/4	1/9	1/4	3/11
B-22	10	7	0/4	1/9	0/4	0/11
B-27	8	6	0/4	0/9	0/4	0/11
B-31	8	8	0/4	2/9	0/4	0/11
B-32	6	5	0/4	0/9	0/4	0/11
B-41	13	10	0/4	2/9	0/4	1/11
B-43	6	5	0/4	0/9	0/4	0/11
B-44	11	7	0/4	1/9	0/4	0/11
B-45	3	4	0/4	0/9	1/4	0/11
B-46	7	6	0/4	0/9	0/4	0/11
Total	79	65				

*Polymorphic data were indicated compared to original plants. A: Non-irradiated variants, B: Irradiated variants. P/T : The number of plants showing polymorphism/ The total number of plants analyzed.

callus was 8.8% (7/79), but that of the regenerates from non-irradiated was not detected compared to the original plant. In White Star, 4 polymorphic bands (6.1%) out of 65 were observed in the plants derived from the irradiated callus. The frequency of variations derived from non-irradiated callus was 3% (2/65) compared to the original plant.

As shown in Table 4, four AFLP primer combinations (E_1-ACG+M_1-CAT, E_1-ACG+M_2-CAA, E_1-ACG+M_3-CAG and E_1-ACG+M_4-CTC) produced a total of 395 and 402 DNA fragments in White Star and Yulmi, respectively. Yulmi regenerates from gamma-treated callus showed a variation of DNA levels with four primer combinations, that is nine out of nine plants in ACG/CAT, ACG/CAA, ACG/CAG and ACG/CTC primer set. The regenerates from the non-irradiated callus revealed a variation for four primer combinations, that is one out of four plants in ACG/CAT, ACG/CAA, ACG/CAG and four out of four plants in ACG/CTC primer set. White Star regenerates from the gamma-treated callus displayed a variation for four primer combinations, i.e. 11 out of 11 plants in ACG/CAT, ACG/CAA, ACG/CAG and ACG/CTC primer set. The regenerates from non-irradiated callus detected a variation with four primer combinations, i.e. 2 out of 4 plants in ACG/CAT primer set, 1 out of 4 plants in ACG/CAA, ACG/CAG and ACG/CTC.

The total polymorphism rate in Yulmi and White Star plants regenerated from irradiated calli were 29.9% (120) and 28.6% (113), respectively, while the frequency of AFLP polymorphisms of Yulmi and White Star somaclones derived from non-irradiated calli were 8.5% (34) and 5.6% (22), respectively. Every primer combination produced polymorphic bands in all variants of Yulmi and White Star.

Two bands detected in the Yulmi original plant were not observed in regenerated variants of Yulmi (small arrows) (Figure 2A). Two bands were observed only in variants derived from the irradiated callus of Yulmi and White Star (big arrows) (Figure 2A, B). Base on the AFLP analysis, the phylogenetic trees produced two main distinct groups in Yulmi and White Star (Figure 3): namely, the conjunction group of original plants (No. 1 in Yulmi

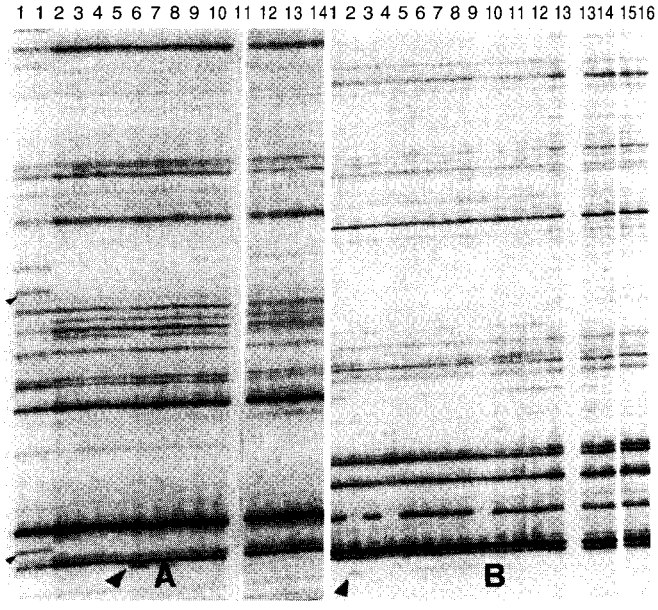

Figure 2. AFLP analysis of sweetpotato cultivars and regenerants. The DNA fingerprints were generated using primer pair E_1+M_4(A) and E_1+M_3(B) to amplify genomic fragments from Yulmi and White Star, respectively.
A: The first two lanes are the Yulmi original plant, the lanes 2 to 10 are irradiated somaclonal variations and the lanes 11 to 14 are non-irradiated somaclonal variations of the same variety.
B: The first lane is the White Star original plant, the lanes 2 to 12 are irradiated somaclonal variations and the lanes 13 to 16 are non-irrdiated somaclonal variations.
Arrow indicates the band of the mutation marker.
Small arrows: That bands were observed in the Yulmi original plant.
Big arrows: That bands were specifically observed in the irradiated variants.

Table 4. Four primer combinations selected for AFLP analysis and polymorphic data *of non-irradiated and irradiated variants in Yulmi and White Star.

Primer Combinations	No. of amplifid bands		P/T			
			Yulmi		White Star	
	Yulmi	White Star	A	B	A	B
E_1-ACG/M_1-CAT	101	99	1/4	9/9	2/4	11/11
E_1-ACG/M_2-CAA	89	90	1/4	9/9	1/4	11/11
E_1-ACG/M_3-CAG	110	108	1/4	9/9	1/4	11/11
E_1-ACG/M_4-CTC	102	98	4/4	9/9	1/4	11/11
Total	402	395				

*Polymorphic data were indicated compared to original plants. A: Non-irradiated variants, B: Irradiated variants. P/T : The number of plants showing polymorphism/ The total number of plants analyzed.

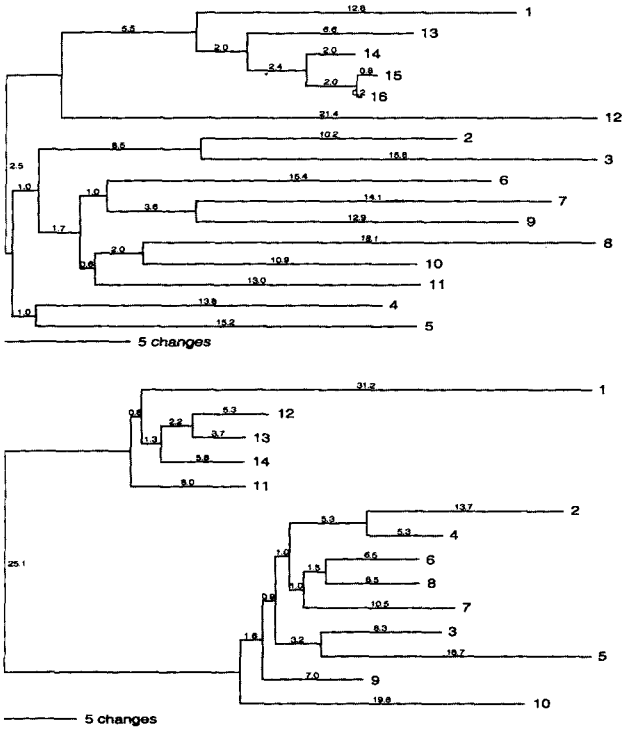

Figure 3. The phylogenetic analysis generated by PAUP 4.0 beta version are showing a plants genetic relationship between the original White Star (top) and Yulmi (bottom), and the plants regenerated from the embryogenic calli irradiated with gamma rays.
Original White Star and Yulmi mother plants (No. 1).
Non-irradiated variants (No. 11, 12, 13 and 14 in Yulmi and No. 13, 14, 15 and 16 in White Star). Irradiated variants (No. 2 to 10 in Yulmi and No. 2 to 12 in White Star).

and White Star) with variants derived from the non-irradiated callus (No. 11, 12, 13 and 14 in Yulmi and No. 13, 14, 15 and 16 in White Star) and the group of variants derived from the irradiated callus, being separated clearly. However, the RAPD-based analysis failed to form the trees into original plants, the non-irradiated and irradiated variants.

Discussion

Formation and embryogenesis of EC were similar to those described by Liu and Cantliffe (1984), yellowish in color, nodular and with compact tissue. However, the finding that EC formation frequency from shoot meristem was higher than those from leaves and petioles was not consistent with the result of Liu and Cantliffe (1984). It seems that the difference in medium components, especially vitamins, and the stage of the young explant used for the culture might be responsible for these differences. The frequency of EC from Yulmi meristems on a medium containing 1 mg/L 2,4-D was similar to that reported by Min et al. (1994), but lower than that (90%) published by Liu et al. (1989 and 1992) in White Star.

In vegetatively propagated plants such as polyploidy sweetpotato, it may be impossible to separate favorable mutations by cross-breeding or back-crossing since these plants may be sterile. And mutagenesis of callus tissue may prove to be more efficient in producing solid mutants (Bhagwat and Duncan, 1998). Therefore, mutation breeding in connection with the *in vitro* method may be necessary in sweetpotato. A useful mutant through this technique was obtained by other authors (Bhagwat and Duncan, 1998; Isabel and Pedro, 1999). In this study, some morphological variations among regenerates derived from non-irradiated and irradiated-calli were observed. These results are in agreement with some reports (Buiatti et al.,1986; Simard et al., 1992). Variations in plant morphology derived from the non-irradiated calli might be due to the influence of the phytohormones contained in the culture medium (van den Bulk et al., 1990) and culture periods (Koornneef et al., 1989). The morphological frequency of variants derived from irradiated callus was higher than that of non-irradiated variants. Thus our results suggest that induced somatic embryogenesis coupled with gamma-rays radiation can lead to an increase in the emergence of variants. Chlorophyll-deficiency was the most frequently observed mutation, and this is consistent with other reports (Karp, 1993; Squillace and Kraus, 1963). Those variants can be used for cloning useful genes and genetic sources. We don't know if such variants are dominant or recessive mutations. However, in most plant species, induced mutations and somaclonal mutations have most frequently been shown to be the results of dominant to recessive mutations, and much of the single gene variation is not seen unless selfed progeny is tested (Godwin et al., 1997).

In this study, RAPD polymorphism was detected in all variants except those from the non-irradiated clones of Yulmi, and the polymorphism among regenerates from the irradiated callus was higher than that from the non-irradiated variants.

Previous studies using RAPD succeeded in the observation of DNA polymorphisms in various species, including *Picea*, *Lolium*, *Hordeum*, and beets (Isabel et al., 1993; Brown et al., 1993; Devaux et al., 1993; Munthali et al., 1996), and were not able to detect polymorphism in apple trees, *Festuca* and *Ginseng* (Mulcahy et al., 1993; Harada et al., 1993; Valles et al., 1993; Shoyama et al., 1997). RAPD analysis has a variety of advantages; it is faster and requires a smaller amount of DNA (Waugh and Powell, 1992), but the reliability of RAPD is limited due to its sen-

sitivity in the PCR condition (Hill et al., 1996). This disadvantage can be overcome by employing the AFLP technique. The results of AFLP analysis were different from those of RAPD in which no polymorphism among regenerates from the non-irradiated callus of Yulmi was detected, but it was observed in the AFLP analysis. Such reasons that AFLP detected a different polymorphic frequency were caused by recognition sites of restriction endonucleases (Vos et al., 1995), insertions and deletions of genomic sequences and the frequency of binding sites of primers. Restriction enzymes that specifically recognizes DNA methylated bases are especially highly heritable in a Mendelian fashion and can produce new alleles at a high frequency in plants (Veilleux and Johnson, 1998). But in RAPD, the ability of sampling a somewhat greater portion of the genome is dependent upon the frequency of the binding sites of the random oligonucleotide primers. Although the different results of AFLP and RAPD were observed, both of them revealed that polymorphism among regenerates from the irradiated callus was higher than that from the non-irradiated, which proves that the combination of radiation with *in vitro* culture can increase the variation frequency in sweetpotato.

The classification of variants based on AFLP and RAPD analyses gave completely different results. The AFLP marker system classified the Yulmi and White Star variants into two clearly distinct groups (non-irradiated vs. irradiated). However, the RAPD analysis was not formed into trees. Based on these results, AFLP analysis is superior to RAPD analysis in classifying variants of sweetpotato.

Consequently, the present data from RAPD, AFLP, and morphological investigation are helpful to establish an efficient irradiation system for *in vitro* mutagenesis to increase mutation spectra and rates. It could be mentioned that plants which show any type of phenotypic variation are expected to show the same at the molecular level. The use of *in vitro* culture methods in combination with radiation techniques seems particularly suitable for the improvement of vegetatively propagated crops (Maluszynski et al., 1995).

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