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Selection and Characterization of S-Aminoethyl-L-Cysteine Resistant Plants from Gamma-ray Irradiated Embryogenic Callus in Sweet potato

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Abstract

Sweet potato cells derived from Yulmi were isolated from embryogenic callus and irradiated with 50 Gy dose. Resistant cells were selected on a MS medium containing 1.0 mM S-aminoethyl-L-cysteine (AEC). This level of AEC approximately inhibits non-selected wild type cells. The callus resistant to this analog of lysine was subcultured for 30 days in absence of AEC to proliferate. The three resistant calli (AR-1, AR-2 and AR-3) with better growth were divvied into 0.5~1mm diameter and placed on MS medium with 0, 0.4, 0.6, 0.8 and 1.0 mM AEC. There are considerable growth difference between control callus and AEC resistant callus on the AEC-medium. The selected calli were placed on the hormone-free medium for regeneration. Three plantlets, five plantlets and six plantlets were recovered from AR-1, AR-2 and AR-3 calli, respectively. Each two regenerants in AR-1, AR-2 and AR-3 were randomly selected for RAPD and SDS PAGE analysis. RAPD polymorphisms between Yulmi and AEC resistant plant from irradiated calli were detected in several Wako primers. Also, it was identified that two AEC resistant plants had higher protein than the original variety Yulmi.

Key words: Embryogenic callus, Induced variation, RAPD, S-aminoethyl-L-cysteine, SDS-PAGE, Sweet potato

Abbreviations: EC (Embryogenic callus), AEC (S-Aminoethyl-L-cysteine), RAPD (Random amplified polymorphic DNA)

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Introduction

The sweet potato (*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). The conventional methods for breeding have some limitations because of the limited genetic resources, and the self- and cross-incompatibility of this crop. So, it was impossible for sweet potato to increase special components such as protein through cross recombination.

When existing germplasm fails to provide the desired recombinant, it is necessary to resort to other sources to induce variation. Combination of radiation techniques with *in vitro* culture methods can speed up breeding programs, from generation of variability, through selection, to multiplication of the new genotypes (Maluszynski et al. 1995). In a wide range of plant species, stable mutant traits have appeared among plants regenerated from *in vitro* mutagenesis in pineapple (Lapade et al. 1995), banana (Matsumoto and Yamaguchi 1991; Raop et al. 1995), and grapevine (Kuksova et al. 1997). This variation was caused by gross alterations in 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).

Important agricultural crops such as sweet potato, rice and corn are nutritionally deficient in lysine. Thus, researchers in agriculture and nutrition need genotypes with the potential for higher lysine production. Cells resistant to the specific inhibitor,

S-aminoethyl-L-cysteine, may provide a mechanism for the recovery of cells with altered lysine metabolism. To obtain lysine overproduction, earlier data with microorganisms indicated that S-aminoethyl-L-cysteine was rather efficient in isolating mutants that accumulate lysine (Nakayama et al. 1966; Demain 1975). Among the few AEC-resistant variants descri-bed in several higher plants were reported to overproduce free lysine (Chaleff and Carlson. 1974; Widholm 1976), but neither genetic and biochemical evidence were made available in support of their results. In other cases strains characterized by altered uptake or incorporation mechanisms were isolated, e.g. in barley (Bright et al. 1979) and in rice (Gideon et al. 1981; Negrutin et al. 1984).

Several strategies can be used to assess the genetic integrity of *in vitro*-derived clones. Among them the PCR-based random amplified polymorphic DNA (RAPD) assay are time and cost-saving analysis method (Williams et al. 1990; Wachira et al. 1995).

The purpose of this study was to select sweet potato tissue cultures resistant to AEC, to confirm DNA variation and the difference of the protein pattern.

Materials and Methods

Plant materials and induction of embryogenic callus (EC)

A sweet potato cultivars, Yulmi, was 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^{-1} sucrose, 4 g l^{-1} phytagel and adjusted to pH 5.7 before autoclaving. In order to induce embryogenic callus, the shoot meristem was taken from 3-week-old *in vitro* plants, 15 explants per 9 cm petri dish, were placed on MS basal medium supplemented with 1mg l^{-1} 2, 4-D, 30 g l^{-1} sucrose, solidified with 4 g l^{-1} phytagel, adjusted to pH 5.7 before autoclaving, incubated in darkness at 27°C and subcultured at 3-week intervals.

Gamma irradiation, selection and regeneration

Embryogenic calli were squashed with the mess and cultured in 120 rpm gyratory shaker for 4 weeks and the suspension was centrifuged to get single cell suspension. It was irradiated with 50 Gy dose from ^{60}Co source in the radiation facility at the Korea Atomic Energy Research Institute, Daejeon, Korea and then cultured for 3 weeks on suspension culture. Cells with a uniform size (300~400 uM) were gathered and inoculated on the solid medium with 1.0 mM AEC about at a final density of 3×10^4 colonies for 30 days. The resistant calli were subcultured on the AEC-free medium to proliferate for 30 days. Then, the proliferated calli were divided into pieces in 0.5~1 mm diameter and inoculated on the medium with various AEC concentration (0, 0.4, 0.6, 0.8 and 1.0 mM) at 25 calli per petri dish with 5 replications for 30 days.

Plantlets were regenerated on 50mL 2, 4-D free-MS medium in a 250mL glass bottle and maintained in a growth chamber at 25±1°C under fluorescent light for 16h/day at 700 μmol /m^2/s. After 30 days *in vitro* culture, the regenerates were acclimatized for 2 weeks in 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.

RAPD analyses

DNA was extracted from the leaves of regenerates, 7 plants with original variety (Yulmi), 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 25μL 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 0.2μM primer. Amplifications were performed with a Perkin-Elmer 480 Thermal Cycler as 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 ethidium bromide. RAPD polymorphic bands were scored as present (1) or absent (0). The Duncan's test and Statistical Packages for Social Sciences (SPSS 1983) were used to compare the original plant and mutants.

SDS-PAGE

One gram of the leaf was obtained from each plant. Protein for SDS-PAGE was extracted in modified Laemmli's (1970) buffer containing 65 mM Tris-HCl pH 6.8, 2% SDS, 5% glycerol, 5% β-mercaptoethanol, 2 mM EDTA. The extracts were boiled for 3 min and microfuged for 5 min. The resulting supernatant containing the total protein was used for SDS-PAGE. Equal concentration (50 μg/ lane) of protein was separated by SDS-PAGE and visualized by staining with Coomassie Brilliant Blue. Protein concentration was determined by Bradford (1976) method.

Results

Embryogenic callus induction and isolation of AEC-resistant cell lines

Within 30 days from inoculation, the embryogenic calli were obtained from the meristem on AEC-free medium. After the squash, the calli were subjected to suspension culture. For the purpose of increasing the selection efficiency, the cells were irradiated with gamma rays (50 Gy). Tolerant ones were selected on the medium of *in vitro* culture with 0.8 mM AEC (Figure 1 A). The calli irradiated with 50 Gy showed better somaclonal potential than non-irradiated calli. Three colonies (AR-1, -2, -3) with better growth were selected and compared in resistance with control calli on AEC-contained medium. They were further confirmed as resistant by a second transfer on selection medium (Figure 2). The frequency of resistant calli at 0, 0.4, 0.6, 0.8 and 1.0 mM AEC appeared significant difference ($P<0.01$) compared to control calli (Figure 2). The resistant calli were cultured on hormone-free medium with 0.8mM AEC, LD_{50} concentration of resistant calli, to regenerate for 40 days. Green spots appeared slowly in many resistant calli (Figure 1 B). A part of calli produced somatic embryos that developed into plantlets after transferred to the hormone-free medium (Figure 1 C).

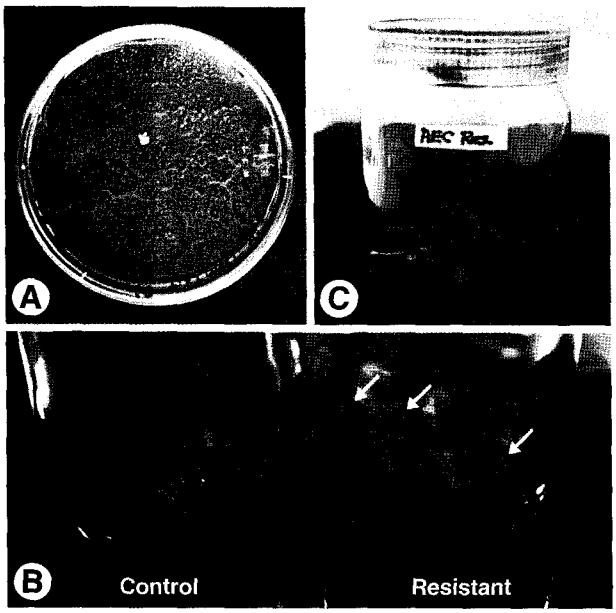

Figure 1. Selection of AEC-resistant callus and regeneration from sweet potato calli irradiated with gamma ray. A, AEC-resistant callus selected on the medium containing 1.0 mM AEC after irradiation with 50 Gy dose; B, Control and resistant calli cultured on hormone-free medium with 0.8 mM AEC for regeneration; C, Plantlets regenerated from the resistant callus. Black arrow: Resistant callus; White arrow: Green spot which were developed to plantlets.

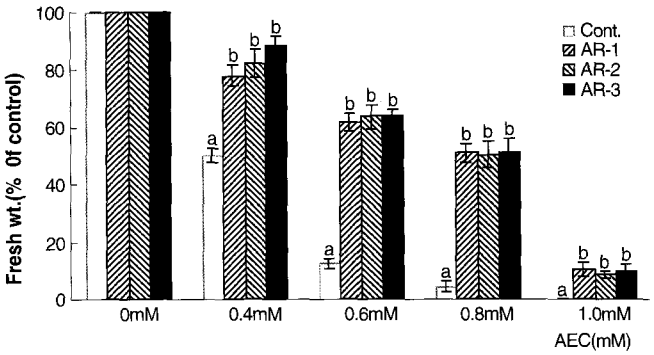

Figure 2. Growth inhibitions of sweet potato control calli and AEC-resistant calli on the medium containing various concentration of AEC. The LD_{50} of non-resistant and resistant calli is 0.4 and 0.8 mM, respectively. Data are means ± SE of 15 independent calli. Bars indicate standard error. a, b: Significantly different within each concentration at P = 0.01 using Duncan's test.

Table 1. The oligonucleotide primers selected for the RAPD analysis and polymorphic data of Yulmi control plant and its AEC-resistants

Wako primers	No. of amplified bands						
	Yulmi	AR-1		AR-2		AR-3	
		-1	-2	-1	-2	-1	-2
C-1	6	6	6	6	6	6	6
C-2	8	8	7	9(1)	8	8	8
C-3	7	7	7	7	7	7	8(1)
C-7	8	7(5)	8(2)	6(2)	8(2)	7(2)	8(2)
C-8	6	5(1)	5(2)	6	6	6	6
C-9	12	12	12	12	11(1)	12	12
C-10	9	9	9	10(1)	9	8(1)	9(1)
C-14	9	9	9	9	9	9	9
C-15	10	10	10	10	11(1)	10	10
C-16	7	7	6(1)	6(1)	7	6(1)	7(1)
Total	82	80	81	81	82	79	83

( ) : Polymorphic band. AR-1, -2, -3: AEC-resistant variants.

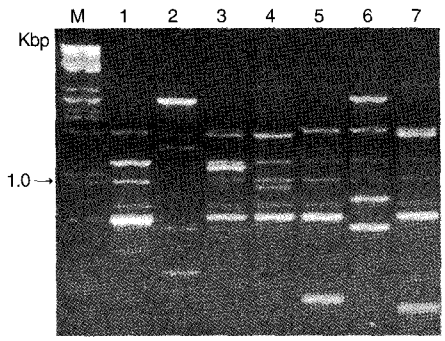

Figure 3. RAPD analysis of sweet potato cultivar, Yulmi, and AEC-resistants. The DNA fingerprints were generated using Wako primer C-07 to amplify genomic fragments from Yulmi and AEC-resistants, respectively. 1: Yulmi control, 2 and 3: AR-1-1, AR-1-2, 4 and 5: AR-2-1, AR-2-2, 6 and 7: AR-3-1, AR-3-2.

Detection of variation

To detect the variation of the regenerates at the DNA level, RAPD analysis was carried out. Ten primers were selected based on their ability to produce reproducible RAPD bands with template DNA from Yulmi and the regenerates, AR-1-1, -2 derived from AR-1 callus, AR-2-1, -2 derived from AR-2 callus, AR-3-1, -2 derived from AR-3 callus. The 10 primers used in the analysis yielded 79~83 bands in Yulmi and regenerates. The number of bands for each primer varied ranging from five for primer C-8 to 13 for C-9. Two primers (Wako C-1, -14) out of ten revealed monomorphic bands in the variants compared to original plant, but the rest produced polymorphism (Table 1). Polymorphic bands for primer C-07 appeared in all variants compared to their original variety (Table 1, Figure 3).

Total protein profiles

Total protein pattern as resolved by SDS-PAGE were very different protein profiles in resistant plants compared with Yulmi control (Figure 4). A density of protein was 2-fold higher in the two resistant plants compared with the parental Yulmi. Interestingly, about a 28~25-kDa protein was present at higher concentration in the mutants (Lane 3, 5) than in the Yulmi (Lane 1). The total proteins of mutants (Lane 2, 7) were present at a lower concentration.

Discussion

Formation and embryogenesis of EC were similar to those described by Liu and Cantliffe (1984) in that yellowish in color, nodular and compact tissue. In vegetatively propagated plants such as polyploidy sweet potato, it may be impossible to separate favorable mutations, such as mutants increasing specific amino acid, 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 *in vitro* method may be necessary to sweet potato. The *in vitro* mutagenesis of cells is important because the technique provides sources of specific variability, which are required for a better understanding of genetic and regulatory phenomena (Widholm 1972). A AEC- mutant through this technique was obtained by another author (Negrutiu et al. 1984). In this study, callus resistant to AEC had unique characteristics. Resistant callus grew more rapidly than controls with inhibitor. This is good evidence that these cell types are genetically unique, heritable and probably mutational changes. The data supports the idea that *in vitro* mutagenesis particularly in the presence of AEC may provide a mechanism to recover cell lines and subsequently plants with the capacity for altered or increased protein and protein lysine in sweet potato. Those variants can be used for cloning useful genes and genetic sources. We don't know if such variants are dominant or recessive mutations in the study. However, in most plant species, induced mutations has 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 are tested (Godwin et al. 1997).

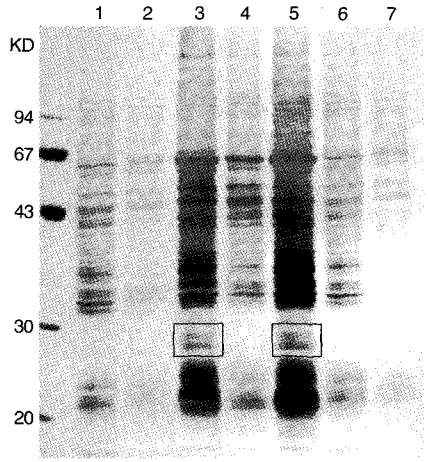

Figure 4. SDS-PAGE analysis of sweet potato proteins. Total proteins were resolved on a SDS-gel and visualized by Coomassie Brilliant Blue. 1: Yulmi control, 2 and 3: AR-1-1, AR-1-2, 4 and 5: AR-2-1, AR-2-2, 6 and 7: AR-3-1, AR-3-2. Proteins in rectangular are more increased than that of Yulmi.

RAPD polymorphism was detected in a part of resistant plants compared with Yulmi. 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 sensitivity in the PCR condition (Hill et al. 1996). Therefore, the reproducible bands with three replications were used to identify polymorphic and monomorphic bands.

The classification of AEC-resistant lines based on SDS-PAGE analysis gave completely different results with Yulmi control in the protein fraction. The SDS-PAGE marker system classified the Yulmi and its two resistant lines. The 25~28-kDa protein was found in higher concentration in the mutants than in the parental Yulmi. Some mutants (lane 3 and 5) in Figure 3 exhibited increased protein content. It is believed that other amino acids as well as amino acid involved in mechanism ana-

log were increased in mutants, which is to prevent the unbalance of the specific amino acid in *in vivo* (Lee et al. 1992). Another features of the two high-lysine maize mutants are that they exhibit altered protein body morphology (Lending and Larkins 1992). In the case of the putative high-lysine sweet potato mutant, it is obvious that it has also undergone alternation in protein body morphology. The protein bodies are more numerous and tightly compacted compared with the Yulmi. It is not known the exact content of lysine in the Yulmi and mutants. So, each free amino acid content analysis will be required to address its distribution in the total protein. Such analysis will also help to monitor any changes in the arrangement of lysine within the protein of mutants. Therefore, the studies on the analysis of each amino acid are yet to be cleared.

Consequently, 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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References

Bhagwat B, Duncan EJ (1998) Mutation breeding of highgate for tolerance to *Fursarium oxysporum* f. sp. *cubense* using gamma irradiation. Euphytica 101: 143-150

Bradford MM (1976) A rapid sensitive method for quantitation of microgram quantities of protein utilization the principle of protein-dye binding. Annual Biochemistry 72: 248-254

Bright SWJ, Fetherstone LC, Miflin BJ (1979) Lysine metabolism in a barley mutant resistant to S-amonoethylcysteine. Planta 146: 629-633

Brown PTH, Lange FD, Kranz E, Lorz H (1993) Analysis of single protoplasts and regenerated plants by PCR and RAPD technology. Mol Gen Genet 237: 311-317

Chaleff RS, Carlson PS (1974) *In vitro* selection for mutants of higher plants. In: Ledoux L (eds) Genetic manipulation with plant material. Plenum Press, New York London, pp 351-357

Demain AI (1975) Overproduction of microbial metabolites and enzymes due to alterations of regulation. In: Ghose TK, Fischer A (eds) Adv Biochem Engine, Vol. 1 Springer, Berline Heidelberg New York, pp 113-142

Devaux P, Kilian A, Kleinhofs A (1993) Anther culture and *Hordeum bulbosum*-derived barley doubled haploids mutation and methylation. Mol Gen Genet 241: 674-679

Godwin ID, Sangduen N, Kunanuvatchaidach R, Piperidis G, Adkins SW (1997) RAPD polymorphisms among variant and phenotypically normal rice somaclonal progenies. Plant Cell Reports 16: 320-324

Harada T, Matsukawa K, Sato T, Ishikawa R, Niizeki M, Saito K (1993) DNA-RAPDs detect genetic variation and paternity in *Malus*. Euphytica 65: 87-91

Hill M, Witsenboer H, Zabeau M, Vos P, Kesseli R, Michelmore R (1996) PCR-based fingerprinting using AFLPs as a tool for studying genetic relationships in *Lactuca* SPP. Theor Appl Genet 93: 1202-1210

Isabel N, Tremblay L, Michaud M, Tremblay FM, Bousquet J (1993) RAPDs as an aid to evaluate the genetic integrity of somatic embryogenesis-derived populations of *Picea mariana* (Mill) B. S. P. Theor Appl Genet 86: 81-87

Kuksova VB, Piven NM, Gleba YY (1997) Somaclonal variation and *in vitro* induced mutagenesis in grapevine. Plant Cell Tiss Org Cult 49: 17-27

Laemmli UK (1970) Cleavage of structural protein during the assembly of the head of bacteriophageT_4. Nature 227: 680-685

Lapade AG, Velug AMS, Santos IS (1995) Genetic improvement of the Queen variety pineapple through induced mutation and *in vitro* culture techniques. FAO/IAEA Intl, Sym on the Use of Crop Improvement, Vienna, 19-23 June 1995, IAEA-SM-340: 145-146

Lee HY, Kim JH, Kim HJ, Kameya T (1992) Selection of rice cell lines resistant to 5-methyltrytophan in cellcultures. Korea J Plant Tissue Culture 19: 289-294

Lee M, Phillips RL (1988) The chromosomal basis of somaclonal variation. Ann Rev Plant Physiol Mol Biol. 39: 413-437

Lending CA, Larkins BA (1992) Effects of floury-2 locus on protein body formation during maize endosperm development. Protoplasma 171: 123-133

Liu JR, Cantliffe DJ (1984) Somatic embryogenesis and plant regeneration in tissue cultures of sweet potato (*Ipomoea batatas* poir.) Plant Cell Rep 3: 112-115

Maluszynski M, Ahloowalia BS, Sigurbjornsson B (1995) Application of *in vitro* mutation techniques for crop improvement. Euphytica 85: 303-315

Matsumoto K, Yamaguchi H (1991) Induction and selection of aluminium tolerance in the banana. IAEA-SM-311/128. pp. 249-255

Mulcahy DL, Cresti M, Sansavini S, Douglas GC, Linskens HF, Bergamini Mulcahy G, Vignani R, Pancaldi M (1993) The use of random amplified polymorphic DNAs to fingerprint apple genotypes. Sci Hortic 54: 89-96

Munthali MT, Newbury HJ, Ford-Lloyd BV (1996) The detection of somalonal variants of beet using RAPD. Plant Cell Rep 15: 474-478

Nakayama K, Tanaka H, Hagino H, Kinosita S (1966) Studies on lysine fermentation. Agric. Biol. Chem. 30: 611-616

Negrutiu I, Cattoir-Reynearts A, Verbruggen I, Jacobs M (1984) Lysine overproducer mutants with an altered dihydrodipicolinate synthase from protoplast culture of *Nicotiana sylvestris* (Spegazzini and Comes). Theor Appl Genet 68: 11-20

Raop S, Ganapathi TR, Bapat VA, Suprasanna (1995) *In vitro* propagation and mutation induction in banana. IAEA-SM-340: 191-192

Schaeffer G W, Sharpe FT (1981) Lysine in seed protein from S-aminoethyl-L-cysteine resistant anther-derived tissue cultures of rice. *In Vtro* 17: 345-352

Shoyama Y, Zhu XX, Nakai R, Shiraishi S (1997) Micropropagation of *Panax notoginseng* by somatic embryogenesis and RAPD analysis of regenerated plantlets. Plant Cell Rep 16: 450-453

Valles MP, Wang ZY, Montavon P, Potrykus I, Spagenberg G (1993) Analysis of genetic stability of plants regenerated from suspension cultures and protoplast of meadow fescue. Plant Cell Rep 12: 101-106

Wachira FN, Waugh R, Hackett CA, Powell A (1995) Detection of genetic diversity in tea using RAPD markers. Genome 38: 201-210

Waugh R, Powell W (1992) Using RAPD markers for crop improvement. TiBtech 10: 186-191

Widholm JM (1972) Anthranilate synthase from 5-methyltryptophan susceptible and resistant cultured *Daucus carota* cells. Biochem Biophys Acta 279: 48-57

Widholm JM (1976) Selection and characterization of cultured carrot and tabacco cells resistant to lysine, methionine, and proline analoges. Can J Bot 54: 1523-1528

Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV (1990) DNA polymorphism amplified by arbitrary primers are useful as genetic marker. Nucleic Acids Res 18: 6531-6535