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GENETIC ANALYSIS OF SOMACLONAL VARIANTS AND INDUCED MUTANTS OF POTATO (*SOLANUM TUBEROSUM* L.) CV. DIAMANT USING RAPD MARKERS

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Abstract

The objective of this work was to genetically analyze somaclonal variants and gamma induced mutants of potato (*Solanum tuberosum* L.) cv. Diamant using RAPD-PCR technique. In the present work, callus was induced from nodes, internodes and leaf explants in MS medium supplemented with NAA (1.0 mg/l) and BAP (0.5 mg/l) and plants were regenerated from 14-20 weeks old calli. For gamma irradiation, ten-week old well proliferating calli were exposed to doses ranging from 5-50 Gy. All the four selected somaclonal variants and five gamma induced mutants were differentiated by banding patterns obtained from 22 primers that generated 140 polymorphic bands. The presence of polymorphic bands in variants and mutants suggest that genetic variation occurred in all the treatments as compared to control. Similarity and clustered analysis were conducted using Jaccard's coefficients and the un-weighted pair-group method using arithmetic averages. The results summarized in a dendrogram, show genetic diversity among the variants and mutants. The study shows that RAPD markers were efficient in discriminating somaclonal variants and induced mutants of potato.

Introduction

Plants regenerated via tissue culture techniques display genetic variations for different characters which have been referred to as somaclonal variations by Larkin and Scowcroft (1981). It is also called tissue or culture-induced variation (Kaeppler *et al*., 2000). Somaclonal variations are generally attributed to pre-existing genetic variation in somatic cells (Walbot, 1985), single gene mutations, aneuploidy, transposable elements, cytogenetic changes and DNA methylation, (Jain, 2001; Gaj, 2004). The extent of variation depends on genotype, age of the donor plant, explant type and plant hormones in the culture medium (Peredo *et al*., 2006). Genetic variability is essential for breeding and selection of superior genotypes for crop improvement (Clegg, 1990). In vegetatively propagated plants such genetic modifications can be directly incorporated into new varieties (Jelenic *et al*., 2001).

In several commercial varieties of potato, tissue culture induced variations were observed in a wide range of characters, such as plant morphology, tuber characteristics (Taylor *et al*., 1993), disease resistance (Matern *et al*., 1978; Behnke, 1979, 1980; Cassells *et al*., 1991; Sebastini *et al*., 1994), isoenzymatic pattern, tuber proteins and chromosome number and structure (Pijnacker & Sree Ramulu, 1990).

Another source of producing variability in plants is by the application of induced mutations. Physical and chemical mutagens have proven useful for increasing the frequency of mutations and variations. Induced mutation techniques have been successfully used to improve yield, quality, and disease and pest resistance in many crops including potato (Ahloowalia 1990; Sonnino *et al*., 1991; Love *et al*., 1993; Das *et al*., 2000; Veitia *et al*., 2001; Rodriguez *et al*., 2002; Yildirim, 2002; Yildirim *et al*., 2003).

A combination of *In vitro* technology and radiation/chemical-induced mutagenesis has been recommended to improve cultivars of vegetatively propagated crops (Novak, 1991; Maluszynski *et al*., 1995). The use of *In vitro* cultures in mutation breeding offers several advantages over the *In vivo* techniques including, obtaining explants from pre-existing cultures and recovering mutants and rapidly micro-propagating them under controlled environmental conditions.

Some of the genetic changes are difficult to observe at the morphological or physiological level because of the structural differences in the gene product that may not alter its biological activity sufficiently to produce an altered phenotype. Analyses of secondary metabolites and isozymes patterns have also been used, but they are limited in their sensitivity (Morell *et al*., 1995). To overcome this problem, DNA markers are conveniently being used to detect tissue culture-induced variations and mutations.

Molecular markers have been used to estimate genetic diversity and examine genetic relationships that exist between cultivars in a range of horticultural crops (Bradley *et al*., 1996; Graham *et al*., 1996; Polzerova, 2001; Yasmin *et al*., 2006; Ehsanpour *et al*., 2007; Joshi & Rao, 2009). RAPD (Random Amplified Polymorphic DNA) analysis using PCR in association with short primers of arbitrary sequences has been demonstrated to be sensitive in detecting variations among individuals (Xena de Enrech, 2000). The advantages of this technique are, a) a large number of samples can be quickly and economically analyzed using only micro-quantities of material; b) the DNA amplicons are independent from the ontogenic expression; and c) many genomic regions can be sampled with a potentially unlimited number of markers (Isabel *et al*., 1993).

The present study was aimed to produce useful variants both by somaclonal variation and gamma irradiation and to analyze the variation at genetic level using RAPD-PCR method.

Materials and Methods

Callogenesis and Organogenesis: Nodal, inter-nodal and leaf explants of cv. Diamant were cultured in MS (Murashige & Skoog, 1962) medium supplemented with phytohormones NAA (1.0 mg/l) and BAP (0.5 mg/l) for the induction of callus. The sub-culturing of main callus was carried out after every 2 weeks in the same medium. Long-term cultures (14-20 weeks) were maintained to get somaclonal variants. For obtaining organogenesis, the calli were transferred to regeneration medium comprising NAA (0.5 mg/l) and BAP (2.0 mg/l) in MS medium. The plantlets thus formed were used to get R1 and R2 generation for further analysis.

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Gamma irradiation: For gamma irradiation, ten week old calli were used. The total doses administered were 5, 10, 15, 20, 25, 30, 40 and 50 Gy. The process of irradiation was carried out at Mark-IV Irradiator at NIAB, Faisalabad. After two weeks of irradiation, the calli from callus induction medium were transferred to regeneration medium. The plantlets thus formed were used to get R1 and R2 generation for further analysis.

DNA isolation: Fresh potato leaves of control plants and selected variants/ mutants were taken for DNA extraction using the modified CTAB procedure (Doyle & Doyle, 1990). Approximately, 100 mg of fresh leaf was ground to a powder in liquid nitrogen, using a mortar and pestle. It was mixed with 2.5 ml CTAB extraction buffer and incubated at 65ºC for 30 minutes. The sample was extracted with 2.5 ml of chloroform / isoamyl alcohol (24:1 v/v) and the aqueous phase was mixed with 2/3 volume of cold iso-propanol. After centrifugation the sample was washed with 0.1 ml cold wash buffer, dried and resuspended in 0.1 ml TE or sterile water. Single stranded RNA was digested with 1.0µg/µl RNase A for 30 minutes at 37ºC and the DNA was quantified by gel electrophoresis.

RAPD analysis: Randomly amplified polymorphic DNA profiles were generated by using 22 primers for the amplification of 10 bp random fragment of genome (Lambda Biotech Inc. 3830 Washington Ave. St Louis MO 63108 USA). PCR reactions were performed in volumes of 25 µl containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin, 50 µM each of dATP, dTTP, dGTP, dCTP , 0.2 µM primer, 0.5 µg of template DNA and 2.5 units of Taq DNA polymerase (Fermentas Inc. 7520 Connelley Drive, Maryland 21076, USA). Amplification was carried out using GenAmp-2700 thermocyler (Applied Biosystems) programmed as follows: 4 minutes at 92ºC, 1.30 minutes at 72ºC followed by 40 cycles of 45 seconds at 92ºC, 1.30 minutes annealing temperature of 28ºC, and a final extension 5 minutes at 72ºC. Amplicons were analyzed by electrophoresis in 1.2% agarose gel run in 0.5% TBE buffer and detected by staining with ethidium bromide. After electrophoresis, the amplified products were viewed under UV transilluminator and photographed (Dolphin-Doc with ID software, Wealtec Corp. USA).

Scoring and analysis of RAPD data: The RAPD data was analyzed using MSVP version 3.1 software. RAPD profile was made on the basis of number of shared amplification products. Similarities were deduced by the presence (1) or absence (0) of a specific DNA fragment. The data was used to generate Jaccard's (Jaccard, 1908) similarity coefficients for RAPD bands and were utilized to generate dendrogram by using un-weighted pair group method of arithmetic means (UPGMA).

Results

Potato plants of cv. Diamant regenerated both from long-term incubated and well-proliferated internode-derived calli and after mutagenic treatments with γ-irradiation were hardened in the glasshouse and were transferred to the field (in tunnels under controlled growth conditions) for obtaining R1 generation. In tunnels, plants were screened on the basis of their morphological characters (data not given). Tubers harvested from selected plants of R1 generation were further grown for R2 generation to confirm the stability of variation under field conditions and four somaclonal variants (SV4, SV5, SV6, SV7) and five gamma mutants (GM7, GM8, GM9, GM10, GM 11) were selected for further analysis.

For RAPD analysis, a total of twenty-two random oligonucleotide primers were evaluated for their ability to prime PCR amplification of potato genomic DNA. The primers revealed 187 clear and easily scorable bands, out of which 140 were polymorphic. The size of the bands that were produced in the PCR reactions ranged from 200-3000 bp, but most of the bands were between 300 and 2000 bp (Fig. 1). The variant SV5 amplified the maximum number of DNA fragments (129) followed by SV7 (118), SV4 (116), GM7 (115), GM8 and GM9 (108), while variant SV6 amplified minimum number of bands (95). The presence of polymorphic bands in variants and mutants suggest that genetic variation occurred in all the treatments as compared to control.

Primer-wise detail of DNA polymorphism detected is given in Table 1. The results given in the Table indicated that quality and quantity of amplification products were sufficient for detection of genetic distance among the potato variants and mutants.

Dendrogram constructed by un-weighted pair group method (UPGMA) with arithmetic averages clustering algorithm from the pair-wise matrix of genetic similarity among somaclonal variants and induced mutants is given in Fig. 2. Different clusters of the dendrogram showed a clear pattern of division among the variants and mutants. Two main groups of clusters were identified.

In the first cluster, SV6 and DT-control are present in one sub-cluster showing genetic similarity while GM10 is present in the second sub-cluster showing genetic distance from them. Second major group consisted of all the rest of the variants and mutants. In this cluster, two sub-clusters comprising SV4 and SV5 and GM9 and SV7 exhibited maximum genetic homogeneity among themselves and clear genetic distances from other mutants, GM7, GM11 and GM8.

Discussion

Tissue culture-induced changes, including morphological, cytological, biochemical and genetic/epigenetic alterations, have been frequently reported in many plants. However, the mechanism underlying this so called somaclonal variation remains largely unclear (Peredo *et al*., 2006; Kaeppler *et al*., 2000). The most common factors affecting somaclonal variation are genotype, explant source, *in vitro* period and culture conditions (Bordallo *et al*., 2004). Unlike epigenetic changes, somaclonal variation which results from altered gene expression is usually irreversible (Karp 1991; 1995). The segregation pattern of mutations in the progeny is mostly Mendelian (Larkin *et al.,* 1984).

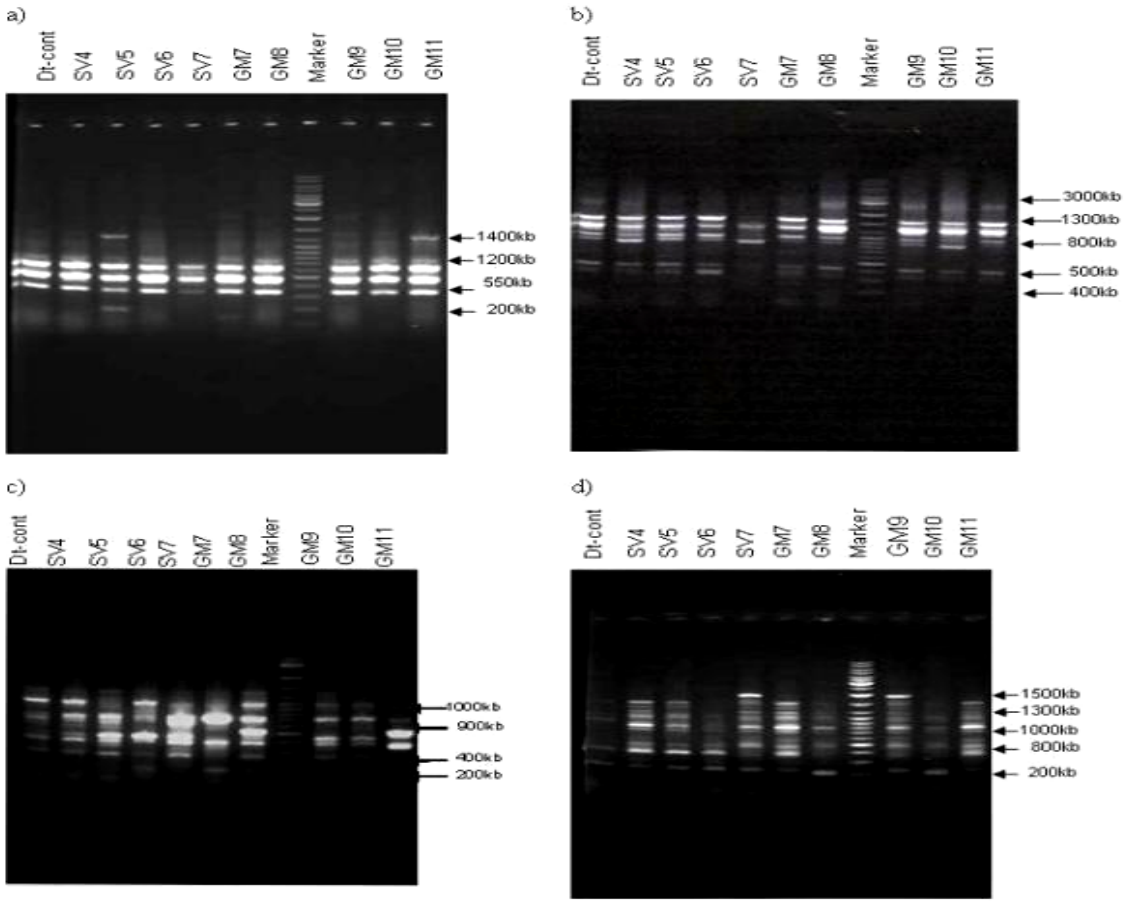

Fig. 1. Amplification products from genomic DNA of selected somaclonal variants and induced mutants of potato cv. Diamant using (a) primer s-10 (b) primer s-109 (c) primer s-68 and (d) primer s-77.

Table 1. Number of monomorphic and polymorphic bands produced by different primers in *Solanum tuberosum* cv. Diamant.

S. No.	RAPD Primer	Primer sequence (5' to 3')	No. of monomorphic DNA fragments	No. of polymorphic DNA fragments	Size (bp)
1.	S-6	5′-TGCTCTGCCC-3′	2	7	200-1200
2.	S-7	5′-GGTGACGCAG-3′	3	5	200-2500
3.	S-10	5′-CTGCTGGGAC-3′	4	8	200-2000
4.	S-18	5′-CCACAGCAGT-3′	2	5	300-1500
5.	S-28	5′-GTGACGTAGG-3′	3	5	300-1200
6.	S-32	5′-TCGGCGATAG-3′	1	7	300-2000
7.	S-33	5′-CAGCACCCAC-3′	1	7	300-2000
8.	S-37	5′-GACCGCTTGT-3′	2	4	400-1200
9.	S-38	5′-AGGTGACCGT-3′	4	3	300-1500
10.	S-44	5′-TCTGGTGAGG-3′	2	4	300-900
11.	S-46	5′-ACCTGAACGG-3′	1	7	200-2500
12.	S-48	5′-GTGTGCCCCA-3′	4	7	200-2000
13.	S-53	5′-GGGGTGACGA-3′	2	7	300-2000
14.	S-57	5′-TTTCCCACGG-3′	2	2	300-1500
15.	S-60	5′-ACCCGGTCAC-3′	1	7	300-2500
16.	S-68	5′-TGGACCGGTG-3′	3	8	300-1200
17.	S-77	5′-TTCCCCCCAG-3′	1	10	200-2000
18.	S-87	5′-GAACCTGCGG-3′	3	8	200-1500
19.	S-99	5′-GTCAGGGCAA-3′	2	8	300-3000
20.	S-104	5′-GGAAGTCGCC-3′	2	6	300-1500
21.	S-109	5′-TGTAGCTGGG-3′	1	8	200-2500
22.	S-114	5′-ACCAGGTTGG-3′	1	7	300-2000

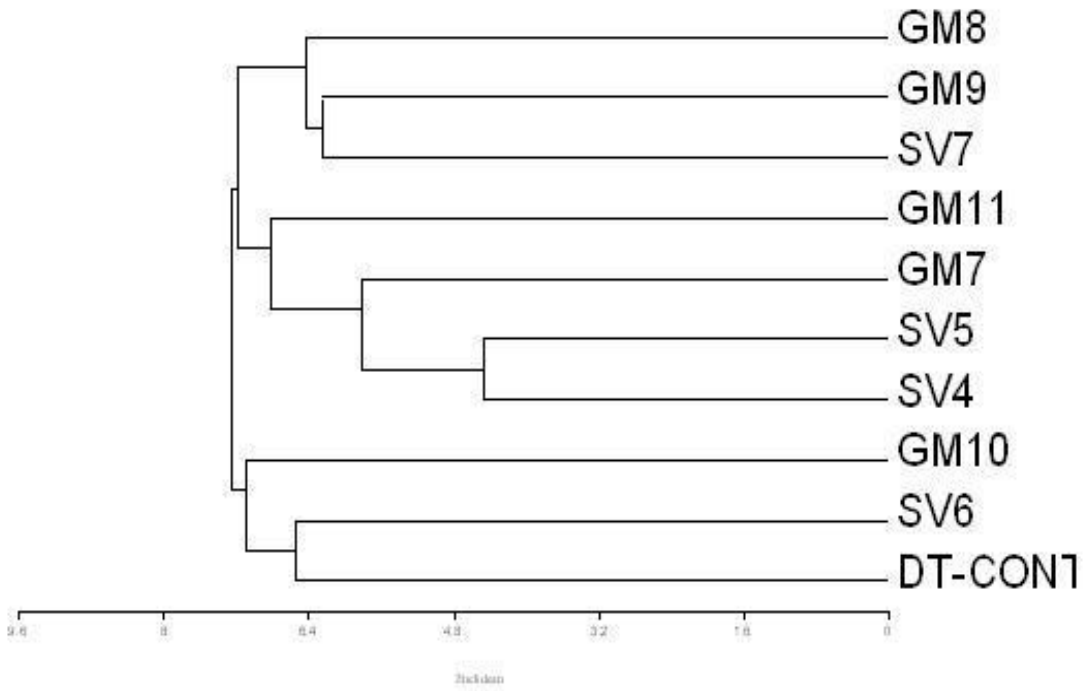

Fig. 2. Dendrogram based on the Jaccard's coefficient, showing the relationships among somaclonal variants and induced mutant of potato cv. Diamant as compared to control.

Physical and chemical mutagens are tools for enhancing and generating genetic variation by inducing changes at the gene, chromosome and genomic levels, in nuclear and cytoplasmic organelle DNA. Induced mutation coupled with *in vitro* propagation technique, has been established as a tool to generate variation in a number of seed and vegetatively propagated crops (Micke, 1991; Donini & Sinnino, 1998). Both somaclonal variation and induced mutations results in the production of new genotypes with a limited change in the original genome. Thus the combination of *in vitro* culture and mutation techniques provides a simple, fast and highly efficient method to improve horticultural crops.

In the present study somaclonal variants and gamma induced mutants were selected on the basis of improved desired characters with special reference to yield potential and agronomic characters (data not given) for further analysis. The importance of somaclonal variation and induced mutations in potato is emphasized by several studies describing the improvements in agronomic characters such as disease resistance, changed tuber shape, skin color, shallow eye, tuber size and yield (Ahloowalia, 1990; Jelenic *et al*., 2001).

Among the molecular markers, RAPD-PCR is being used successfully to identify, characterize and estimate genetic divergence of potato cultivars (Moisan-Thiery *et al.,* 2001; Rocha *et al*., 2002). In this study RAPD-PCR method was used to detect genetic variability among the somaclonal variants and induced mutants of potato as compared to control. Four somaclonal variants and five gamma induced mutants of potato cv. Diamant regenerated from internode-derived calli were genetically analyzed.

Forapani *et al.,* (1999) and Moisan-Thiery *et al*., (2001) identified respectively, 37 and 57 potato genotypes using only three primers. Demeke *et al*., (1993) identified 36 commercial potato cultivars using the RAPD technique with only two primers while Ghislain *et al*., (1999) using 12 primers obtained 102 polymorphic markers, in the discrimination of 128 accesses of andigena potato. Similarly Collares *et al.,* (2004) efficiently characterized 27 potato genotypes on the basis of RAPD markers. In our study, 22 arbitrary primers were used producing 187 bands, out of which 140 were polymorphic, and these primers produced more fragments and more amplified polymorphism as compared to these authors.

For detecting genetic variation among the somaclonal variants among five genotypes of potato and their somaclonal variants, Bordallo *et al*., (2004) also used RAPD-PCR technique. Likewise Wendt *et al.,* (2001) used 10 RAPD primers for the molecular analysis of gamma irradiated regenerated plants of potato cv. Macaca. The primers produced 70 fragments being 24% polymorphic.

Conclusion

The present study clearly shows that variations are present in the plants regenerated via somaclonal variation and induced mutations and also suggested that the RAPD methodology is efficient in detecting somaclonal variation and induced mutations in potato.

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