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Analysis of polymorphism based on SSCP markers in gamma-irradiated (Co^{60}) grape (*Vitis vinifera*) varieties

D. Değirmenci Karataş[1], B. Kunter[2], G. Coppola[3] and R. Velasco[3]

[1]Wine Production Technologies Programme, Bismil Vocational College, Dicle University, Diyarbakır, Turkey
[2]Department of Horticulture, Faculty of Agriculture, Ankara University, Ankara, Turkey
[3]Istituto Agrario San Michele all'Adige, Research Center, San Michele all'Adige, Trento, Italy

This study is part of a PhD thesis presented by D.D. Karataş.
Corresponding author: D.D. Karataş
E-mail: dilek76@gmail.com

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ABSTRACT. The effects of induced mutation produced by five different doses of gamma irradiation (20, 25, 30, 40, and 45 Gy) were determined using molecular approaches in *Vitis vinifera* cultivars, namely Thompson Seedless (Sultani Çekirdeksiz) (progenitor of seedless *vinifera* variety) and Kalecik Karası (one of the best quality wine grape variety of Turkey). Mutant candidates were selected through morphological observations of mutation-induced phenotypic changes during the first, second and third vegetation periods after radiation applications. Amplification studies started with 50 primers (expressed sequence tags) applied to the mutated individuals. Only 15 of these primers revealed polymorphic profiles. Twenty-two candidate mutants of Thompson Seedless and Kalecik Karası, selected based on morphological observations, were analyzed with 15 single-strand conformation polymorphism (SSCP) markers, together with 46 control plants. Polymorphic bands were rarely obtained in the SSCP analysis, and they were not reproducible.

Key words: SSCP; *Vitis vinifera* L.; Mutation; Gamma radiation

INTRODUCTION

Mutations are defined as heritable changes in the DNA sequence that is not derived from genetic segregation or recombination (Van Harten, 1998). Current scientific and technical advances at present can induce mutations with new possibilities to contribute to plant improvement. Spontaneous and induced mutations have also already played an important role in the development of fruit cultivars. Induced mutation breeding could change one or more important traits of grapevine and could therefore play an important role in isolating interesting traits for grapevine breeding (Predieri, 2001). In grapevine breeding programs, sources of new varieties are derived by the selection of somatic mutants and classical hybridization. Currently, there are many somatic mutant varieties that are important for commercial production (Moretti, 1983; Fregoni, 1998, 2000). Lately, new mutated grape varieties have been developed in Russia and in Italy. The mutant variety "Fikreti" is derived from "Marandi" in Russia. In Italy, several mutants were developed from Banarda, Regina Vigneti and Dolcetto cultivars (Maluszynski et al., 2000).

The use of molecular markers in the selection stage of breeding studies has become very important. RAPD, SSR, AFLP, and SSCP (single-strand conformational polymorphism) markers have been used for the genetic discrimination of mutated individuals (Scott et al., 2000; Herrera et al., 2002). SSCP markers have been widely applied in medical diagnosis by human genetics (Jafri et al., 2004; Lee et al., 2005). SSCP markers have also been used for the determination of the molecular heterogeneity of viruses in grapevines (Goszczynski and Jooste, 2002). However, few studies have been reported in terms of plant genetics (Wang et al., 2001; Sato and Nishio, 2003; Salmaso et al., 2004). Rather than obtaining DNA sequence data, it is less expensive and faster to use techniques that estimate sequence variations. SSCP analysis has an advantage because of its sensitivity and informative results on mutation detection (Sunnucks et al., 2000). SSCP analysis was first described in 1989 (Orita et al., 1989) as a new approach for detecting DNA polymorphisms or sequence variations. SSCP analysis offers an inexpensive, convenient and sensitive method for determining genetic variation (Sunnucks et al., 2000; Shirasawa et al., 2004).

The aim of the present study was to discriminate genetic polymorphisms between gamma-irradiated (Co^{60}) individuals of Thompson Seedless and Kalecik Karası grape varieties (*Vitis vinifera* L.) using SSCP markers at the Molecular Biology Laboratory of Istituto Agrario San Michele all'Adige (Trento, Italy).

MATERIAL AND METHODS

Gamma radiation was applied to increase variation within the population of the Thompson Seedless and Kalecik Karası grape varieties. For this purpose, research materials were irradiated using the Co^{60} gamma source at the Turkey Atomic Energy Corporation and Sarayköy Nüclear Education Center.

Five different doses (20, 25, 30, 40, and 45 Gy) of gamma radiation were used. For each dose, 100 single bud canes were irradiated for both cultivars, and thus, a total of 1000 single bud canes were irradiated. Nurseries were obtained by grafting irradiated single bud canes on 99R rootstock. After gamma radiation treatments, some losses due to the physiological damages caused by the effect of environmental and radiation effects occurred, but the remaining healthy individuals were planted in the research parcel. Therefore, the initial population included 207 plants for Thompson Seedlees and 315 plants for Kalecik Karası.

Phenotypic characters observed with the naked eye were recorded, and morphologic changes were determined in comparison with control plants at the first (M1V1), second (M1V2) and third (M1V3) vegetation periods upon radiation. The effects of induced mutation produced by 5 different doses of gamma irradiation (20, 25, 30, 40, and 45 Gy) were determined at the DNA level using molecular markers on the *V. vinifera* L. cultivars Thompson Seedless and Kalecik Karası. Mutant candidates were selected through morphological observations of the mutation-induced phenotypic changes. For both Thompson Seedless and Kalecik Karası varieties, a total of 46 candidate mutant individuals that had typical morphological differences (shortest nodium, twin bud formation, large leaf formation, and chlorophyll mutations) were selected among the gamma-irradiated (Co^{60}) population of two grape varieties.

DNA extraction and SSCP analysis

DNA isolation studies were done according to the method of Lodhi et al. (1994) at the Molecular Biology Laboratory, Ankara University, Faculty of Agriculture. For polymorphism detection in selected mutants by the SSCP technique, the strategy of primer selection is necessary. For this reason, studies started selecting genes for the detection of polymorphism in candidate plants. Previously, 50 genes were selected based on the homology with transcription factors, mainly chosen among those that are homologous to genes responsible from morphological characters by screening NCBI (National Center for Biotechnology Information) and Istituto Agrario San Michele all'Adige databanks. In order to detect polymorphisms, analysis was carried out with these selected primers on selected candidate groups. Expressed sequence tags (ESTs) were developed by IASMA (genomics.research.iasma.it). The EST list of primers generated from IASMA is presented in Table 1. Each EST name refers to: INFIO, flower; GEMMA, bud; RADIC, root; BACCA, berry; GERMO, shoot, and FOGLIO, leaf.

Primer design

Primer design was carried out using the GeneRunr 3.4 software, allowing the prevention of hairpin loops and dimers. It is possible to get a desired primer melting temperature as well. Amplification studies started with 50 primers (ESTs) in the selected group of mutated individuals.

Of 50 primers, only 15 primers revealed a polymorphic profile. Thus, these selected 15 primers (RADIC 118, RADIC 294, RADIC 561, RADIC 1104, RADIC 1188, RADIC 1517, GEMMA 1026, GEMMA 1097, GERMO 220, GEMMA 243, GEMMA 334, GERMO 890, INFIO 432, INFIO 622, FOGLIO 236) (Salmaso et al., 2004; Moser et al., 2005; Troggio et al., 2007) were used for further SSCP analysis on all 46 selected candidate individuals.

SSCP analysis

SSCP electrophoresis (Orita et al., 1989) was carried out on a non-denaturating gel as reported by Salmaso et al. (2004). In order to carry out a selective DNA amplification, specific conditions were applied as follows: 2 µL DNA (10 ng/µL) was mixed with 2.5 µL 10X buffer (Qiagen), 2 µL dNTPs (10 mM), 1 µL forward and reverse primers (forward primer, 10 µM), 0.2 µL Taq-

Table 1. Expressed sequence tag (EST) list of primers that were used for single-strand conformational polymorphism (SSCP) studies, generated from IASMA (genomics.research.iasma.it).

N	EST name	NCBI number	Species name	Primer	Sample
1	ISMAAEST000037	15236109	*Arabidopsis thaliana*	INFIO 160	Flower
2	ISMAAEST000066	22023157	*Oryza sativa*	GERMO 322	Shoot
3	ISMAAEST000221	30694805	*Arabidopsis thaliana*	RADIC 1892	Root
4	ISMAAEST000369	15223290	*Arabidopsis thaliana*	GEMMA 967	Bud
5	ISMAAEST000562	15240297	*Arabidopsis thaliana*	RADIC 114	Root
6	ISMAAEST000679	24850307	*Oryza sativa*	RADIC 1637	Root
7	ISMAAEST000695	15227754	*Arabidopsis thaliana*	RADIC 1517	Root
8	ISMAAEST000771	30024598	*Lotus corniculatus*	INFIO 727	Flower
9	ISMAAEST001401	21593586	*Arabidopsis thaliana*	BACCA 672	Berry
10	ISMAAEST001581	15242784	*Arabidopsis thaliana*	BACCA 1409	Berry
11	ISMAAEST001618	15223618	*Arabidopsis thaliana*	GERMO 220	Shoot
12	ISMAAEST001692	18407554	*Arabidopsis thaliana*	RADIC 1104	Root
13	ISMAAEST001697	30696297	*Arabidopsis thaliana*	GEMMA 1097	Bud
14	ISMAAEST001706	30696193	*Arabidopsis thaliana*	BACCA 023	Berry
15	ISMAAEST001728	30908921	*Oryza sativa*	RADIC 1188	Root
16	ISMAAEST001748	15222161	*Arabidopsis thaliana*	BACCA 048	Berry
17	ISMAAEST001954	4760692	*Nicotiana tabacum*	BACCA 1500	Berry
18	ISMAAEST001968	22331031	*Arabidopsis thaliana*	BACCA 1016	Berry
19	ISMAAEST002050	6552389	*Nicotiana tabacum*	RADIC 1187	Root
20	ISMAAEST002110	15228188	*Arabidopsis thaliana*	INFIO 0620	Flower
21	ISMAAEST002140	30677923	*Arabidopsis thaliana*	RADIC 0493	Root
22	ISMAAEST002216	5917653	*Petroselinum crispum*	RADIC 1731	Root
23	ISMAAEST002241	15237721	*Arabidopsis thaliana*	RADIC 294	Root
24	ISMAAEST002248	15242272	*Arabidopsis thaliana*	GERMO 209	Shoot
25	ISMAAEST002275	18396143	*Arabidopsis thaliana*	GERMO 379	Shoot
26	ISMAAEST002276	15239413	*Arabidopsis thaliana*	GEMMA 243	Bud
27	ISMAAEST002380	15222223	*Arabidopsis thaliana*	BACCA 135	Berry
28	ISMAAEST002738	15239113	*Arabidopsis thaliana*	GEMMA 334	Bud
29	ISMAAEST002745	26451690	*Arabidopsis thaliana*	INFIO 410	Flower
30	ISMAAEST002781	15222433	*Arabidopsis thaliana*	RADIC 930	Root
31	ISMAAEST002840	15240297	*Arabidopsis thaliana*	RADIC 046	Root
32	ISMAAEST002850	18423250	*Arabidopsis thaliana*	GERMO 28699	Shoot
33	ISMAAEST002862	30680980	*Arabidopsis thaliana*	INFIO 192	Flower
34	ISMAAEST002892	20127075	*Arabidopsis thaliana*	GERMO 307	Shoot
35	ISMAAEST003027	15240604	*Arabidopsis thaliana*	INFIO 432	Flower
36	ISMAAEST003084	15240754	*Arabidopsis thaliana*	INFIO 340	Flower
37	ISMAAEST003172	30695456	*Arabidopsis thaliana*	RADIC 811	Root
38	ISMAAEST003203	28629811	*Arabidopsis thaliana*	FOGLIO 236	Leaf
39	ISMAAEST003268	15233516	*Arabidopsis thaliana*	RADIC 138	Root
40	ISMAAEST003358	30024600	*Lotus corniculatus*	RADIC 1845	Root
41	ISMAAEST003447	15240708	*Arabidopsis thaliana*	INFIO 622	Flower
42	ISMAAEST003450	15248520	*Arabidopsis thaliana*	RADIC 305	Root
43	ISMAAEST003566	20466590	*Arabidopsis thaliana*	GEMMA 1639	Bud
44	ISMAAEST003637	15236725	*Arabidopsis thaliana*	GEMMA 1026	Bud
45	ISMAAEST003691	7528276	*Mesembryanthemum crystallinum*	BACCA 1688	Berry
46	ISMAAEST003734	15228188	*Arabidopsis thaliana*	GERMO 890	Shoot
47	ISMAAEST003740	25354704	*Arabidopsis thaliana*	RADIC 561	Root
48	ISMAAEST004035	11282608	*Arabidopsis thaliana*	RADIC 1075	Root
49	ISMAAEST004118	11273985	*Arabidopsis thaliana*	INFIO 135	Flower
50	ISMAAEST003915	2573367	*Arabidopsis thaliana*	RADIC 118	Root

polymerase (Qiagen) (5 U/µL) and H_2O to give a final volume of 25 µL. DNA was amplified under the following thermal cycling conditions: one cycle for 5 min at 95°C, 30 s at 94°C, annealing for 1 min at 57°C, 1.5 min for extension at 72°C, and a final extension at 72°C for 15 min.

In order to visualize polymerase chain reaction (PCR) products on an agarose gel, 5-µL DNA samples were loaded along with 1.5 µL Syber Gold and 2 µL loading buffer on a 1.5% agarose gel. In order to quantify PCR products the Mass ruler DNA ladder (Low range; Fermentas, Life Sciences) was used. The gel was stuck to one glass plate by 25 µL γ-

methacryloxypropyltrimethoxysilane (Sigma M65-14) and 15 µL acetic acid in 5 mL ethanol (100%). The second plate was covered with repel-silane ES (Plus One, Amersham). In order to prepare the acrylamide gel solution, 7.5 mL acrylamide (MDE Gel solution ideal for heteroduplex and SSCP analysis, Biospa), 3 mL glycerol, 1.8 mL TBE (10X), 150 µL APS, 18.8 µL TEMED (Plus One, Amersham) were dissolved in 17.7 mL water. Nine microliters formamide was added to 5 µL PCR product; after denaturation (95°C for 2 min), for 6 of these, 14 µL was loaded on an acrylamide gel. The gel was run for 16 h at 135 mV. After electrophoresis, the gel was stained and developed in order to visualize bands of interest. The conditions were as follows: in fixative solution (EtOH, acetic acid, H_2O) for 5 min, then staining solution (EtOH, acetic acid, $AgNO_3$), and finally transferred to the developing solution (NaOH, formaldehyde) for 10 min. Images were acquired by the Adobe Photoshop software.

RESULTS

In SSCP analysis, polymorphic bands were rarely obtained and were not reproducible. In assessing the results obtained from SSCP analysis, the occurrence of mutation in tiny areas of genome as expected in mutant candidates and unknown genetic source of mutations are the two main hardships in obtaining polymorphism after SSCP analysis.

The reason for obtaining very little polymorphism by many researchers intending to determine clonal variation in natural mutant candidates is that mutations, depending on a variety of research, occur in very tiny areas of the genome with unknown sources of clonal variations. It was also emphasized by these researchers that different primer combinations and marker combinations were efficient (Cervera et al., 1998; Scott et al., 2000; Fanizza et al., 2003).

At the end of SSCP analysis, it was concluded that 14 primers (of 15 available) showed monomorphic bands and that only one primer (RADIC 294) revealed polymorphic bands with TS 25 Gy 43 mutated sample (Figure 1). However, when it was repeated to check for polymorphism, it was monomorphic.

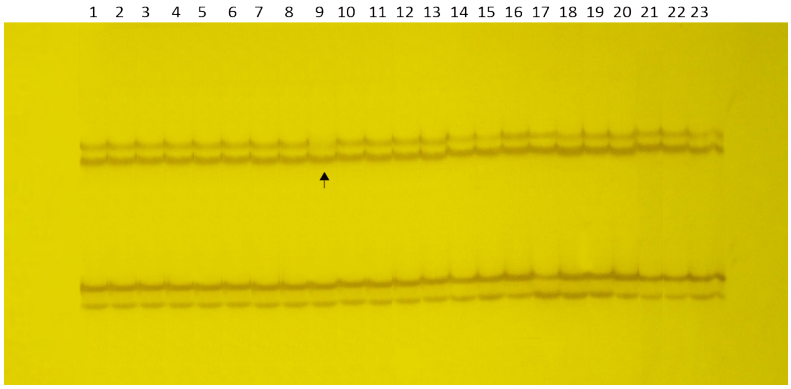

Figure 1. SSCP results with RA 294 primer on TS 25 Gy 43. *Lane 1* = Control; *lane 2* = TS 20 Gy 29; *lane 3* = TS 20 Gy 46; *lane 4* = TS 20 Gy 69; *lane 5* = TS 25 Gy 2; *lane 6* = TS 25Gy 12; *lane 7* = TS 25 Gy 14; *lane 8* = TS 25 Gy 40; *lane 9* = TS 25 Gy 43; *lane 10* = TS 25 Gy 44; *lane 11* = TS 25 Gy 48; *lane 12* = TS 25 Gy 60; *lane 13* = TS 25 Gy 61; *lane 14* = TS 30 Gy 2; *lane 15* = TS 30 Gy 5; *lane 16* = TS 30 Gy 9; *lane 17* = TS 30 Gy 11; *lane 18* = TS 30 Gy 14; *lane 19* = TS 30 Gy 34; *lane 20* = TS 30 Gy 41; *lane 21* = TS 30 Gy 44; *lane 22* = TS 40 Gy 21; *lane 23* = TS 40 Gy 24. Note: The band indicated by an arrow belongs to the TS 25 Gy 43 mutated individual.

DISCUSSION

SSCP marker to discriminate mutations is a method widely used, especially for the diagnosis of diseases in medicine and for SNP definitions. Although there are insufficient studies on plants, SSCP markers have been used especially with the aim of carrying out mapping studies in *V. vinifera* in recent years (Salmaso et al., 2004; Moser et al., 2005; Troggio et al., 2007).

SSCP methods provide a great advantage in defining nucleotide variations without the need for the analysis of DNA sequences (Orita et al., 1989). The nucleotide changes in the DNA sequence amplified with related primers affect the electrophoretic mobility of the DNA forming a different banding pattern (Anonymous, 2005). Because of being gene-specific SSCP markers, information of gene sequence for primer design has been used to define the mutations (Hayashi, 1992). The most important factor that affects the success of SSCP analysis is the size of the related gene (Nataraj et al., 1999).

In the current study, 22 candidate mutants of Thompson Seedless and Kalecik Karası, together with those selected based on morphologic observations and cytological investigations, were analyzed with 15 SSCP markers together with control plants, comprising a total of 46 plants. However, all individuals revealed monomorphic bands with the 15 SSCP primers used. During primer selection, due to insufficient knowledge of genes that control important special features of grapevines, by means of homologous genes, which were responsible for vegetative characteristics, SSCP primers were selected to distinguish genetic polymorphisms to designate mutant candidates. When results obtained from SSCP analysis were designated, SSCP showed monomorphic bands in mutant candidates:

- Occurrence of mutation is in very tiny areas within the genome.

- Amplified area in gene zone coding for determined morphologic characteristics is very small.

- Due to insufficient knowledge of genes controlling the phenotypic specialities of grapevines, the number of primers used for SSCP is fairly limited.

The results of this study are important in two ways. First, by transferring mutants obtained by artificial mutation techniques into vineyard conditions; an important source of material is generated for grapevine breeding programs. Second, the utilization of SSCP markers to determine polymorphisms among this novel mutant population is a new and original approach in our country. The individuals and findings obtained in the current study can be used to generate novel mutant individuals, and may also serve as a source to provide genetic background and variation that can be used for functional analysis and genetic mapping studies, an important aspect in plant breeding.

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