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Establishing Genetic Relation between a Marker Locus and Fungal Disease in Grapevine Using Multidimensional Scaling Analysis

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Abstract

In a hybrid population of 'Italia' (female) and 'Mercan' (male) grape cultivars with their 60 offsprings, a classical two-point linkage analysis was attempted with 300 RAPD, 20 SSR and 24 AFLP primers using Mapmaker/Exp 3.0. Linkage groups of 12 in 'Italia' and 4 in 'Mercan' were attained with 25 and 8 marker loci, respectively. Resistance phenotypes and segregating characteristics were scored as quantitative traits in two growing seasons. MDS analysis was performed to examine the relationship between the marker loci and the fungal diseases, powdery and downy mildew, both of which developed naturally on the progenies. Results of the MDS showed that five and eight marker loci were in close relationship with the powdery and downy mildew, respectively. The R^2 was 62.12%, which considered as acceptable fit and the stress coefficient was 0.26.

Keywords: grapevine, linkage analysis, fungal disease

Introduction

Breeding for disease resistance is one of the major goals in viticulture. Classical breeding to produce resistance genotypes requires a long-term dedication from crossing to the release of a new cultivar. Analysis of genetic relationships in crop species is an important component of crop improvement programs, as it serves to provide information about genetic diversity and is a platform for stratified sampling of breeding populations. Analysis of genetic diversity in germplasm collections can facilitate the stages in reliable classification of accessions and identification of core accessions with possible utility for specific breeding purposes (Mohammadi and Prassanna, 2003).

In a genetic system, most of the characters are associated with each other, and such association may be the product of some pleiotropic effects of gene, existence of two genes on the same chromosome, chromosomal segmental affiliation or due to environmental influences (Rothwell, 1983). Therefore, information of character association between traits and disease resistance component is important to select high resistant genotypes.

Basic correlations between many traits and characters with high priority have been made in grapevine for *Botrytis cinerea* (Gabler *et al.*, 2003), *Plasmopara* resistance (Kortekamp and Zyprian, 2003), vineyard performance (Carey *et al.*, 2007) and for metabolite profiling of flavonols and anthocyanins (Mattivi *et al.*, 2006). Bhatti (1972) contradicted that correlation studies alone do not clearly disclose reliable information for selection and inadequate knowledge of interrelationship of heritable traits may lead to negative results.

The objective of this study was to develop and evaluate molecular markers linked to two fungal diseases (downy and powdery mildew) of grapevine for their utilization in screening resistant or susceptible genotypes.

Materials and methods

Mapping population

The construction of the map was based on the study of 60 progenies from a cross between 'Italia' and 'Mercan' parents. 'Italia' is a standard table grape cultivar (*Vitis vinifera* L.) grown in many regions of the world. 'Mercan' is a white, small berried juice variety grown in the Black Sea region and thought to be a *Vitis labrusca* variety. It is believed to be a genetic resource for tolerance to mildew diseases due to the *labrusca* lineage. These cultivars were crossed in 1992 to obtain genotypes possessing fungal disease resistance. The parents and the derived progenies were grown on their own roots at Tekirdağ Viticultural Research Institute, Turkey.

Phenotypic evaluation of resistance traits

Two fungal diseases (powdery mildew and down mildew) were observed and scored for two consecutive years at Tekirdağ Viticultural Research Institute-Turkey. Resistance of the F_1 plants to powdery mildew and downy mildew was determined by the method of Eibach (1994) and IPGRI, UPOV, OIV (1997), respectively. The 'Italia' parent was rated 5 (highly susceptible), whereas the 'Mercan' parent was scored 1 (highly resistant) to both diseases.

Genotyping

Cuttings obtained from the institute was potted in perlite:soil:peat moss (1:1:1) in a greenhouse located at the Department of Horticulture, Faculty of Agriculture, Ankara University. DNA was extracted from young leaves with a modified CTAB-based method (Lodhi *et al.*, 1994). DNA quantification and purity were determined with NanoDrop ND1000 (NanoDrop Technologies, Wilmington, DE, USA).

For RAPD analysis, 10-mer primers from Kits A, B, C, D, E, F, H, K, M, N, and O, together with I (1-16), P-17, G-5 and G-6 (Operon Technologies, Alameda, Calif. and Roth, Karlsruhe, Germany), UBC series (204, 231, 237, 238, 251), BC series (302, 340, 374), B-352, B-356, B-379, B-389, B-392, P-33, P-35, P-123, P-166, P-210, P-232, P-250, P-255, P-313, P-325, P-382, P- 394, P-402, P-437, P-443, Kozak primers (1-8), GT-04, S-34, S-35, S-39 and S-69 (Research Genetics, Huntsville, Ala.) and from a RAPD series (1-9), OPU-16 and SC series (1022, 1023, 1038, 1043, 1048, 1059, 1065, 1076, 1077, 1082, 1093) from IDT (Integrated DNA Technologies, Inc., Coralville, Iowa) were used in 25 µl reaction mixture containing 100-200 ng template DNA, 1.5 u Taq DNA polymerase (Promega, Wis.), 0.25 mM of each of four dNTPs, 0.2 µM oligonucleotide primers 10-17 bases long, 500mM KCl, 100mM Tris-HCl (pH 9.0 at 25°C) and 1% Triton® X-100. The reaction mixture was overlaid with a drop of mineral oil. Amplification was performed on a thermocycler (PTC-100; MJ Research Inc., Waltham, Mass.) for 35 cycles at 94°C for 30 s, 35°C for 60 s and 72°C for 105 s followed by a final cycle of 8 min at 72°C. The amplification products were resolved on 1% agarose plus 1% Nusieve™ (FMC Corp., Maine) agarose gels. Electrophoresis was carried out in 1X TBE (Tris-Boric acid-EDTA) buffer (Sambrook *et al.*, 1989) at 7 V/cm. The RAPD bands were visualized using 0.01 mg ml^{-1} ethidium bromide under UV light (λ=302 nm) and recorded on Type 65 Polaroid film. Polymorphic RAPD bands were scored as either present or absent.

SSR and AFLP primers were synthesized at Raffi Miskciyan, IDT (Integrated DNA Technologies, Inc., Coralville, IA, USA). γ^{33} P was synthesized at Izotop (Institute of Isotopes Co., Ltd., Budapest). Multiplication of 24 AFLP combinations (Tab. 1) and 20 SSR primers developed within the *Vitis* Microsatellite Consortium (VMC). [VVMD32, VVMD26, VVMD5, VVMD6, VVMD7 (Bowers *et al.*, 1996); VVMD28, VVMD36, VVMD31, VrZAG29, VVMD17 (Bowers *et al.*, 1999); VVS3, VVS5, VVS1, VVS4, VVS2, VVS29 (Thomas and Scott, 1993); VrZAG21, VrZAG79, VrZAG112, VrZAG67 (Sefc *et al.*, 1999)] was applied.

SSR reactions were performed in a 25-µl reaction mixture containing 10-ng template DNA, 10 pmol of each primer, 0.25 mM of each of four dNTPs, 0.5 u Taq DNA polymerase (Promega, WI, USA) and 1.5 mM $MgCl_2$. Amplification conditions were optimized individually for each marker. The amplifications were performed using a thermocycler (PTC-100; MJ Research Inc., Waltham, MA,USA) programmed as follows: 4 min at 94°C followed by 36 cycles of 1 min at 94°C, 1 min at 56 or 60°C, 1 min at 72°C followed by a final hold of 8 min at 72°C. All amplifications were confirmed by running 5.0µl of the PCR reaction product on 2% agarose gels. PCR products were mixed with 2x sequencing dye (98% formamide, 10 mM EDTA, 0.05% bromophenol blue and xylenecyanol), denatured at 94°C for 2 min and 3 µl was loaded onto 6% polyacrylamide gels and run in1x TBE buffer at 70 W for 3 h. Gels were visualized by silver staining technique (Promega, Wis., USA).

AFLP markers were generated using the protocol described by Vos *et al.* (1995) with the following modifications. 500 ng genomic DNA was digested with EcoRI (10u) for 3 h at 37°C, and Tru 9 (5u) for 3h at 65°C. DNA was linked to EcoRI (Pharmacia) and MseI (New England Biolabs) adaptors with T4 DNA ligase (1u) for 12 h at 16°C. After 10 times dilution pre-amplification was performed by 20 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 60 s followed by an extension reaction for 72°C for 60 s. The selective amplification was carried out under the following conditions: 1 cycle at 94°C for 30 s, 65°C for 30 s and 72°C for 30 s, followed by 12 cycles in which annealing temperature decreased 0.7°C per cycle. Additionally 23 cycles followed at 94°C for 3 s, 56°C for 30 s and 72°C for 60 s. EcoRI primer was endlabelled with [γ33 P] ATP. After selective amplification, AFLP products were mixed with equal volume (10 µl) of denaturation dye (98% formamide, 10 mM EDTA, 0.025% bromophenol blue, 0.025 Xylene Cyanol) and denatured by 95°C for 5 min. 3 µl of each sample were loaded and electrophoresed on 6% sequencing polyacrylamide gel and run in 1x TBE buffer at 70 W for 3 h. The gel was dried with a vacuum dryer and exposed to X-ray film (Kodak Biomax MS Film, MS-2 Size: 35 cm x 43 cm) for autoradiography at room temperature between 2-4 days.

Scoring, data organization and analyses

The amplification products were scored from negatives as either present or absent. Data were entered in Microsoft® Excel (Microsoft Corp.) spreadsheets.

Linkage analysis was performed with MAPMAKER/EXP 3.0 (Lander *et al.*, 1987; Lincoln *et al.*, 1992). For each parental data set, all markers were individually evaluated by the chi-square method to detect, if any, deviation of gametic segregation from the expected Mendelian 1:1 ratio ($p \leq 0.05$). Two independent data sets were generated that separately contained the meiotic segregation information from each parent. In the absence of phase information, each segregating locus was paired with a dummy locus, resulting in a double data set (double pseudo-testcross strategy; Weeden *et al.*, 1994). Segregating markers were placed into linkage groups under high stringency of LOD 5.0. Linkage groups obtained from the doubled data were then divided into two symmetrical sets of groups, and one set was chosen for further analysis (Lodhi *et al.*, 1995).

MDS-(multi dimensional scaling) analysis

Due to the relatively low number of marker loci that placed on the linkage groups, QTL analysis could not be performed. MDS analysis was performed to examine the relationship between the marker loci and the fungal diseases, powdery and downy mildew, which developed naturally on the progenies. We chose to use MDS because this method assumes no statistical distribution assumptions (Schiffman *et al.*, 1981).

Results

To select primers generating polymorphic bands, 'Italia' and 'Mercan' DNA were amplified using 300 RAPD, 20 SSR and 24 AFLP primers. Two hundred and twenty-three of the clearly amplified markers followed a 1:1 segregation ratio when tested by χ^2 test: 131 in 'Italia' and 92 in 'Mercan' (Tab. 1). Among these markers were 117 RAPD, 16 SSR and 90 AFLP. Only 31 markers (11 RAPD, 6 SSR and 14 AFLP) were mapped in 16 linkage groups in both parents: 12 in 'Italia' and 4 in 'Mercan'. Total genetic distance covered at high stringency (min. LOD5.0 and max. distance of 25 cM) was 249.52 cM for 'Italia' and only 31.6 cM for 'Mercan'. Mean distances between the markers were 9.6 and 3.95 cM for maternal and paternal parent, respectively. Distances between the adjacent markers ranged from 1.7 to 25.5 cM for 'Italia' and 0.0 to 15 cM for 'Mercan'.

Non-metric MDS analysis by Euclidean distance model of the markers that were mapped in the linkage groups revealed that 14 markers in two clusters were in association with powdery and downy mildew resistance (Fig. 1). The markers associated with powdery mildew were in a cluster of three RAPD markers (OPM2A from the paternal parent), and three AFLP markers (from the maternal parent). On the other hand, three RAPD, two SSR and three AFLP markers from the maternal parent were in the same cluster that was linked with downy mildew.

Tab. 1. Contribution of the various marker types segregating at 1:1 ratio to the maps of 'Italia' and 'Mercan'

Marker type	Mapped in 'Italia'	Mapped in 'Mercan'	Total
RAPD	62	55	117
SSR	11	5	16
AFLP	58	32	90
Total	131	92	223

RSQ (*Squared correlation index,* R^2) value was 62.12%, with $R^2 >= 0.60$ considered acceptable fit. The stress factor, the proportion of the variance of the disparities not accounted for by the MDS model, was 0.26 which was considered as a fair fit in terms of goodness-of-fit (Kruskal, 1964). The stress value becomes smaller as the estimated map distance approaches the original distance (Mohammad and Prasanna, 2003).

Discussion

This study was conducted to establish a relationship between fungal disease resistance and three different types of molecular markers on a segregating population of grapevine cultivars, 'Italia' and 'Mercan.' Downy mildew (*Plasmopora viticola*) and powdery mildew (*Uncinula necator* (Schw.) Burr.) are the main fungal diseases affecting grapevines, especially cultivars of *Vitis vinifera* L.

After the first genetic map of grapevine (Lodhi *et al.*, 1995) was completed, many researchers have been focused their studies on finding genetic relationships between markers and fungal disease resistance (Luo *et al.*, 2001; Fischer *et al.*, 2004; Barker *et al.*, 2005; Gökbayrak *et al.*, 2006; Akkurt *et al.*, 2007; Welter *et al.*, 2007; Işçi *et al.*, 2010). They were all able to utilize mapping programs specifically developed to find the relationships because of the high number of marker loci that placed on linkage groups.

In the event that these specific programs cannot be utilized due to the low number of marker loci placed on linkage groups, multivariate analysis proves to be a big help. MDS is a procedure that represents a set of individuals or genotypes (n) in a few dimensions (m) using a similarity/distance matrix between them such that the inter-individual proximities in the map nearly match the original similarities/distances (Johnson and Wichern, 1992). There are some studies in which researches used this type of statistical tools. Esti *et al.* (2010) applied correspondence analysis on elicitation frequencies of wine description and reported that the results were preliminary but useful tool for identifying and selecting 'Grechetto' wine attributes. Parr *et al.* (2007) investigated the distinctive New Zealand wine style 'Marlborough Sauvignon blanc' by sensory characterization and chemical analysis of selected aroma compounds using MDS. Calonnec *et al.* (2004) used MDS to successfully group batches of 'Cabernet Sauvignon' according to powdery mildew disease severity. Everhart *et al.* (2008)

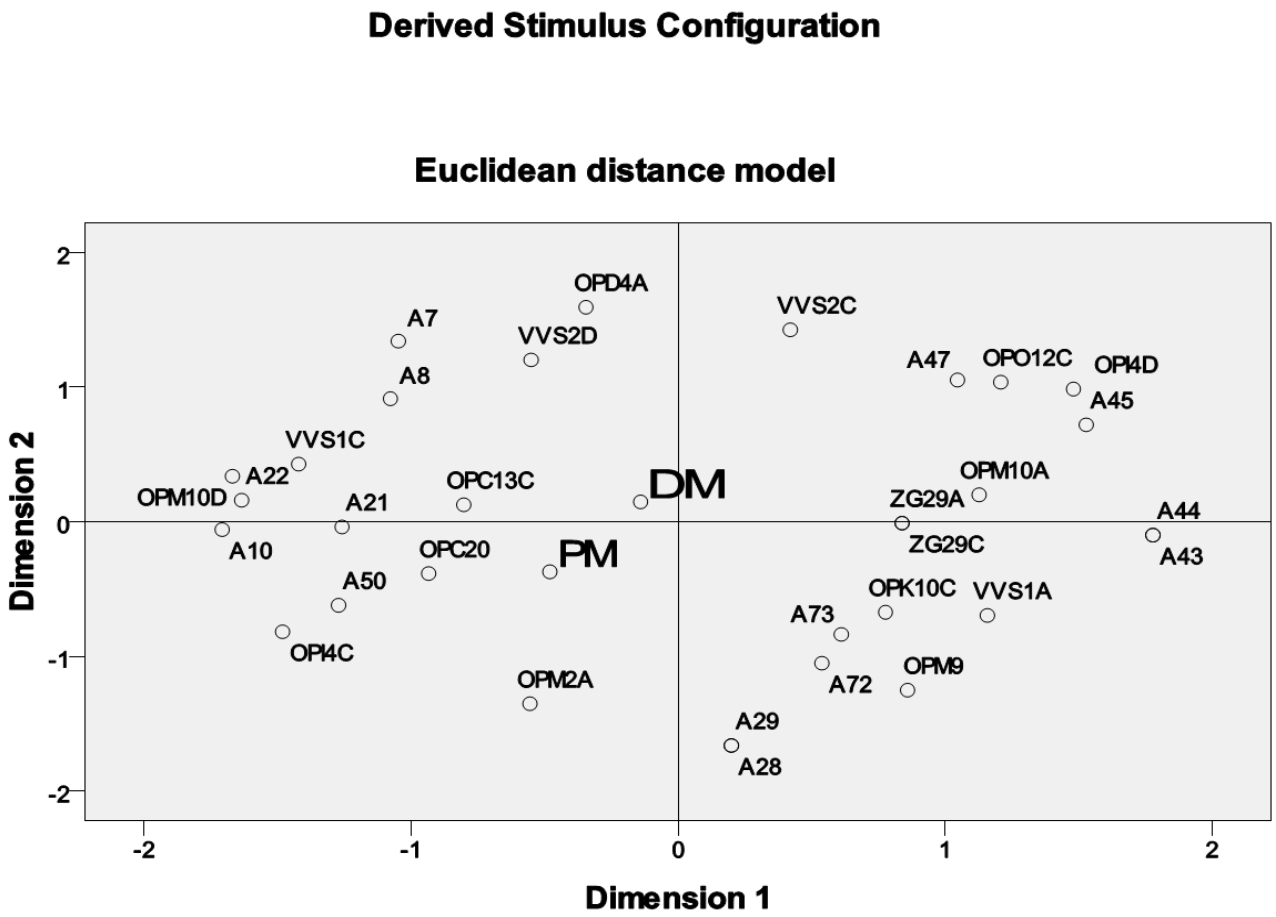

Fig.1. Molecular markers represented in multidimensional space for their relations to powdery and downy mildew of grapevine population ('Italia' x 'Mercan')

used non-metric multidimensional scaling to explore the possible relationships of myxomycete species and sample sites among all measured environmental factors for grapevine species.

The total genetic distance covered in the present study at this high stringency was very low for both of the parents, showing very low frequency for both maternal and paternal meiotic recombination. The distance covered here is not in the range of those reported for grapevine (Lodhi *et al.*, 1995; Dalbo *et al.*, 2000; Doligez *et al.*, 2002; Grando *et al.*, 2003). This might have been the result of poor selection of segregation population, low heterozygosity and low number of progeny under investigation.

Choice of properly resistant parent in a susceptible x resistant cross is very important. Regner *et al.* (2003) reported that only a few plants out of a big population from a cross of susceptible x resistant genotypes could reach the same or similar level of resistance as the donor parent. Only by chance genotypes carrying all relevant parental genes could be found. Therefore, our donor of mildew resistance ('Mercan') appears to be an improper donor in screening seedlings in a marker assisted selection.

The genetic map was constructed based on the data gained from our present research using MAPMAKER program (Lander *et al.*, 1987; Lincoln *et al.*, 1992) with min LOD5.0 and max. distance of 25 cM. We only gained 12 and 4 linkage groups for maternal and paternal parent, respectively. This showed us that we have to increase the number of vines and markers to the level where the amplification of parental alleles can take place and be detected. The first two authors of this paper carried out separate mapping studies using RAPD and SSR-ALP primers on the same plants (Gökbayrak *et al.*, 2006; Işçi *et al.*, 2010). Gökbayrak *et al.* (2006) found that only one RAPD marker from maternal plant were found in significant relationship with powdery mildew, whereas İşçi *et al.* (2010) found two SSR markers in close relationship with it. Both researchers did not detect any marker-downy mildew relation. When all three different types of molecular markers were combined and searched for their proximities to the powdery and downy mildew resistance genes, markers found in relationship differed, indicating the necessity of using increased number of markers to obtain reproducible healthy results.

Grapevine breeders dealing with acquiring resistance to fungal diseases have to pay attention that resistance is controlled by polygenes, field response of mature vines does not always correlate well with the seedlings or their

cultivation under artificial conditions, and results obtained through artificial infections may not be solidified in the intact vines. These highlight the importance of confirming resistance across multiple geographic regions with the use of uniform testing procedures on different breeding populations.

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