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ORIGINAL PAPER

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EVALUATION OF GRAPEVINE RESISTANCE TO DOWNY AND POWDERY MILDEW IN 'REGENT' × 'BOĞAZKERE' HYBRID POPULATION SEGREGATING FOR RESISTANCE GENES

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ABSTRACT

Downy (*Plasmopara viticola*) and powdery mildew (*Erysipha necator*) is known as one of the most mischievous diseases for viticulture in Turkey as well as in the world. Therefore breeding studies play an important role for development of new cultivars resistant against fungal diseases. The aim of this research was to develop new F_1 population and evaluate the resistance of hybrids to powdery and downy mildew via marker assisted selection (MAS). Resistant 'Regent' and susceptible 'Boğazkere' varieties were used to obtain hybrid population. A total of 6 markers belonging to 3 genomic regions were used for DNA based selection. Four SSR (UDV15, VMCNG2f12, VMC7F2 and UDV305) and two SCAR (ScORNA7-760 and ScORN3-R) markers which were developed to the resistance loci of Rpv3 and Ren3 were used for DNA based selection. The results were evaluated together with powdery mildew inoculation observations. When inoculation observations and MAS were evaluated, genotypes resistant, tolerant and susceptible to powdery and downy mildew were identified. Especially 16 genotypes identified as resistant to powdery mildew, can be used in future breeding programs.

Key words: breeding, marker-assisted selection, grapevine, 'Boğazkere', 'Regent', powdery mildew

INTRODUCTION

Grapevine (*Vitis vinifera* L.) is one of the most important fruit crops domesticated in the northeast part of Turkey according to archaeological findings [McGovern 2003]. 'Boğazkere' (*Vitis vinifera* L.) is a Turkish wine grape variety originated from Diyarbakir Province. 'Regent' was bred by the "Institute for Grapevine Breeding Geilweilerhof" as a new cultivar resistant to fungal diseases [Eibach et al. 2003].

Fungal pathogens are known as one of the important problems in cultivation of grapevine around the world. Powdery mildew [*Erysiphe necator* Schwein. (syn. *Uncinula necator* (Schw.) (Burr.)] and downy mildew [*Plasmopara viticola* (Berk. and Curtis) Berl. and de Toni] are the two most significant diseases endangering the grape production worldwide [Wan et al. 2007]. *Erysiphe necator* produces whitish mycelia on the surface of leaves, stems, inflorescences and berries. Both diseases can progress rapidly and cause serious economic losses [Calonnec et al. 2004, Blanc et al. 2012].

Vitis vinifera is very common cultivated grapevine species in the world. All current cultivars of *V. vinifera* have been accepted as sensitive to downy and powdery mildew diseases, although susceptibility varies among

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cultivars [Kono et al. 2015]. Control of mildew infections on traditional *V. vinifera* cultivars requires regular application of chemicals, and growers tend nowadays to limit the risk of epidemics by applying large amounts of fungicides. However, routine use of fungicides has high risk on human health. The use of fungicides is also expensive and has negative environmental effects. Furthermore, breeding the resistant species decreases the efficiency of these sprays on strains of *P. viticola* in the vineyard [Furuya et al. 2010].

Plant breeding for disease resistance appears to be a helpful way to effectively control grapevine downy and powdery mildew and in an environmentally friendly manner. Therefore, the use of molecular markers is becoming more and more important for breeding purposes. Developing the molecular markers linked to powdery and downy mildew traits, which was strongly supported by sequencing of various genomes, allows the application of MAS.

The availability of different types of molecular markers has significantly facilitated researches breeding *Vitis* species in last years. Appropriate screening methods for disease resistance and fruit quality are necessary for efficient breeding programs. The existence of several types of molecular markers, and differences in their fundamentals and implementations require attentive consideration in choosing one or more of such methods.

These techniques increase the scope and precision of predictions of the outcomes of breeding decision to breeders. It helps to identify parents that are suitable for cross breeding and to pre-screen the resistance population for precise traits and separate those accessions with the undesirable quality, before planting in the vineyard. The use of molecular markers in plant breeding is called marker-assisted selection (MAS) and is a part of the new discipline of "molecular breeding" [Bertrand et al. 2007].

Marker assisted selection in grapevine breeding as a new tool allows applicants to increase the breeding efficiencies. The results illustrate that MAS offers a rapid and accurate method to select hybrid genotypes that combine multiple loci of interest in grape. In this way, undefined progeny can be eliminated and the size of the hybrid population can be reduced early during the breeding process [Di Gaspero and Cattonaro 2010].

The aim of this research was to combine classical hybridization study with marker assisted selection (MAS) technique. In this breeding study, we used 'Regent' carrying the dominant (*Ren*3) and (*Rpv*3) resistance genes as a paternal, also 'Boğazkere' as a susceptible maternal cultivars to achieve 169 F_1 population. 4 SSR (UDV305, UDV15, VMC7F2, VMCNG2F12) and 2 SCAR (ScORN3-R, ScORA7-760) markers were used for identifying the resistant genotypes.

Another goal of our study is to increase the effectiveness of our resistance breeding program via marker assisted selection and to evaluate the molecular markers, which are related to the resistance loci from different resistance sources. Our results verify decisive step towards Marker Assisted Selection (MAS) in grape breeding programs.

MATERIALS AND METHODS

Plant material. In the study, 'Regent' resistant to mildew diseases and the susceptible 'Boğazkere' cultivars were used as parents. The distinction of resistance to powdery and downy mildew was investigated in 169 F_1 progenies of the cross 'Regent'× 'Boğazkere'. 'Regent' was created in 1967 by Professor Gerhardt Alleweldt at the Institute for Grapevine Breeding Geilweilerhof by hybridizing Diana with a Silvaner × Müller-Thurgau cross and this *Vitis vinifera* variety was combined with the interspecific hybrid Chambourcin (Chancellor × Villard blanc-S.V.12-417) [Alleweldt and Dettweiler-Munch 1992, Akkurt et al. 2007]. At present it is among the most important new fungal-resistant quality grape variety world-wide, especially in German wine regions.

It shows high-range resistance to both fungal diseases of *P. viticola* and *E. necator* in the field. 'Regent' pedigree was supposed to involve seven distinct wild species several generations back that potentially carried resistance genes of (*V. aestivalis*, *V. berlandieri*, *V. cinerea*, *V. labrusca*, *V. lincecumii*, *V. riparia* and *V. rupestris*) [Akkurt et al. 2007]. 'Boğazkere' is the most popular red wine grape variety in Turkey which is susceptible to powdery and downy mildew. 'Boğazkere' originated from Diyarbakır province near the Tigris river in the southeastern region of Turkey. Crossing and nursing of F_1 population was done in a greenhouse of Ankara University, Faculty of Agriculture, Department of Horticulture in Turkey.

Table 1. Rating levels for oidium infection of leaves of genotypes for their degree of resistance to oidium under the same conditions

Level	Symptoms
1	Very low (tiny spots or no symptoms; neither visible sporulation nor mycelium)
3	Low (limited patches < 2 cm diameter; limited sporulation and mycelium; the presence of *Uncinula* is only indicated by a slight curling of the blade
5	Medium (patches usually limited with a diameter of 2–5 cm)
7	High (vast patches; some limited; strong sporulation and abundant mycelium)
9	Very high (very vast unlimited patches or totally attached leaf blades; strong sporulation and abundant mycelium)

DNA extraction. Young leaves were picked from the shoots and after shock-freezing in liquid nitrogen stored at −80°C until use. In order to obtain genomic DNA, 80 to 100 mg of fresh and healthy leaves of plants were ground in liquid nitrogen to a fine powder and processed according to [Lefort et al. 1998]. The concentration and purity of each DNA sample was checked on agarose gels prepared in TBE buffer. DNA samples that gave a smear in the gel were thrown away, thus the DNA extraction protocol was repeated for these samples. The concentration of each DNA sample also was determined spectrophotometrically at 260 nm (the average yield of DNA was 20 ng L^{-1}, as measured by absorbance at 260 nm using a NanoDrop ND-1000 model spectrophotometer, Nano-Drop Technologies, Wilmington, DE, USA). The quality of DNA was checked by running 5 µL of DNA in 1% (w/v) gels in 1× TBE buffer. The final DNA concentration was adjusted to 15 ng/µl and stored at −20°C.

Natural infection. The experiments were carried out according to common procedures and no pesticides were used in the experimental area. All vines were potted (minimum three plants per cultivar with three replicates) and exposed to natural infection. Powdery mildew infected leaves and shoots were distributed randomly within the plants to provide sufficient inoculation. The natural infection evaluation was carried out on leaves and clusters during the summer period. Four or five young leaves were selected from the top of each vine and were examined at different times during the summer period for disease symptoms. Powdery mildew infection severity on leaves was determined based on the percent of disease spots observed on the entire leaf area according to the procedure described in Table 1 [GENRES-081 1997].

Molecular marker analysis

SSR Analysis. Genomic DNA from hybrid plants was amplified by PCR with selected four SSR primers in a Biometra T-1 Thermocycler (Biometra, Göttingen, Germany). Amplification was carried out in a reaction volume of 10 µl containing 15 ng of DNA, 0.5 mM dNTP, 20−50 pmol of each forward and reverse primer, 0.4 unit GoTaq DNA Polymerase (Promega, Madison, WI) that includes 1.5–3.5 mM $MgCl_2$. PCR conditions had an initial predenaturation step at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing for 1 min at 50°C–60°C (depending on each primer pair-specific annealing temperature) and 1 min at 72°C with a final extension at 72°C for 10 min.

PCR products were diluted with SLS (sample loading solution) at certain ratios according to the fluorescent dyes used in labeling, followed by the addition of Genomelab DNA Standard Kit-400 and electrophoresed in CEQTM8800 capillary DNA analysis system (Beckman Coulter, Fullerton, CA). Allele sizes were determined for each SSR locus using Beckman CEQ fragment analysis software. Allele sizes of 'Regent' were used as reference in each fragment analysis.

All four SSR primer pairs (VMCNG2F12 [Bellin et al. 2009], UDV305 [Bellin et al. 2009], UDV15 [Welter et al. 2007] and VMC7F2 [Riaz et al. 2011]) were used to amplify the resistance loci related to powdery and downy mildew diseases. The size of the alleles obtained from F_1 plants, associated with resistance genes were performed as compared with them.

SCAR Analyses. Two SCAR primers linked resistant loci *Ren*3 were used for screening of 169 F_1 genotype. PCR reactions were performed a total volume of 20–25 µl containing 0.25 mM of each dNTP, 0.25 µM of each primer, 0.5U Taq DNA polymerase, 1.5 mM MgCl2, and 20–40 ng template DNA. The PCR conditions were as follows: an initial of pre-denaturation cycle of 94°C for 4 min, followed by 30–35 cycles of denaturation at 94°C for 1 min, annealing for 1 min, a synthesis step at 72°C for 2 min, and final extension at 72°C for 10 min. Amplification products were separated on a 1.5–2% agarose gel. DNA bands were visualized under UV light (λ = 312 nm) after staining with Ethidium Bromide (0.5 µg/ml) and documented with Genius Bio imaging system.

Data analyses of all markers were evaluated for all 169 seedling of 'Regent' × 'Boğazkere'. Alleles for the SCAR markers (ScORN3-R and ScORA7-760) were scored as dominant markers to prove the powdery and downy mildew diseases with a designation of "1" specifying the presence of an expected amplification product and "0" indicating the absence of an expected amplification product according to Eibach et al. [2007] and Akkurt et al. [2007].

RESULTS AND DISCUSSION

In this research, we analyzed our breeding result of 169 hybrids from the cross of 'Regent' × 'Boğazkere' grape cultivars against to fungal downy and powdery mildew diseases via Marker Assisted Selection. To find resistance genotypes via MAS, firstly we evaluated 4 SSR (UDV305, UDV15, VMC7F2 and VMCNG2F12) markers to follow the allele sizes of markers in grape genotypes.

Table 2. Allelic distributions of 'Regent', 'Boğazkere' and hybrid genotypes with 5 SSR markers (resistance correlated allele sizes are showed as italics)

Cultivars	Markers			
	UDV305	UDV15	VMC7F2	VMCNG2F12
'Regent'	175:189	206:212	159:161	229:299
'Boğazkere'	177:193	201:205	151:181	301:304
Hybrid genotypes	175/177	201/206	159/151	229/301
	175/193	205/206	159/181	229/304
	177/189	201/212	151/161	299/301
	189/193	205/212	161/181	299/304

Table 3. Selection of downy and powdery mildew resistant genotypes by MAS

Marker	Locus	Allele size associated with resistance (bp)	Resistant genotypes (number)
UDV305	Rpv3	299	35
VMCNG2f12	Rpv3	159	37
VMC7F2	Rpv3	206	70
UDV15	Ren 3	189	102
ScORNA7-760	Ren3	760	54

Welter et al. [2007], identified *(Rpv*3) resistance locus of *Plasmopora viticola* by interval mapping in 'Regent' for the first time. *Rpv3* is located on chromosome 18 and confers a 6 hypersensitive response against *P. viticola* strains that carry the same type of disease factor [Bellin et al 2009, Riaz et al. 2011, Di Gaspero et al. 2012, Zini et al. 2015]. Di Gaspero et al. [2012] reported that the allele size of "299" bp from UDV305 SSR marker is known as a resistance linked allele. We received 229:299 bp allele size from resistant parent 'Regent' and 301:304 bp allele size from susceptible parent of 'Boğazkere' (Tab. 2). 35 genotypes of F_1 carry 299 bp allele size, which means that they can be resistant to downy mildew. These 35 genotypes were selected as resistant candidate against *P. viticola* (Tab. 3). VMCNG2F12 marker was also linked withRpv3 locus, on chromosome (chr) 18 and related to downy mildew resistance. Eibach et al. [2007] found that 159 bp allele size indicated the resistance locus of Rpv3. In our research, we amplified 159:161 bp allele sizes in 'Regent', also in 37 F_1 plants from 'Regent' × 'Boğazkere' population (Tab. 2). According to the results obtained from the VMCNG2F12 marker, these 37 genotypes were selected as genotypes that could be resistant to *P. viticola* (Tab. 3).

VMC7F2 is a different marker that is related with multiple disease resistance and also seedlessness. Bellin et al. [2009] explained a single dominant allele at the Rpv3 locus on chromosome 18 in 'Bianca' 2.9 cM apart between UDV305 and VMC7F2 markers for downy mildew resistance. Also VMC7F2 primer was associated with both downy mildew (Rpv3 locus) and powdery mildew resistance (Ren4). Bellin et al. [2009], Riaz et al. [2011], Guillaumie et al. [2013] reported that "206" bp known as a resistant allele for VMC7F2 primer. In our research 'Regent' carried 206:212 bp alleles as resistance parent. We estimated that 206 bp alleles could be resistance correlated allele from 'Regent'. Therefore we got 70 F_1 progenies that carry 206 bp allele as candidate powdery mildew resistant genotypes (Tabs. 2 and 3).

UDV15 marker was used as a major resistance locus against powdery mildew (Ren3) [Eibach et al. 2007, 2010, Welter et al. 2007]. Zini et al. [2015] have used UDV15 marker for Ren3 resistance that is on chromosome 15. We obtained 175:189 bp allele sizes from 'Regent' and 177:193 bp allele sizes from 'Boğazkere' (Tab. 2). According to personal information received from Eibach, 189 bp allele is Ren3 related allele and in our progenies 102 F_1 genotypes showed 189 bp allele size. Therefore, this 102 progenies were selected as candidate genotypes resistant to powdery mildew (Tab. 3).

SCAR markers ScORA7-760 and ScORN3-R were developed from the genetic map of 'Regent' as powdery mildew resistance related markers from major QTL region of linkage group 15 [Akkurt et al. 2007]. Eibach et al. [2007], reported that 760 bp allele size from ScORA7 is related to powdery mildew resistance in the crossing progeny of VRH3082-1-42 × 'Regent'. These markers were used in our crossing population to find the resistance candidate genotypes via Marker-Assisted Selection. Using ScORA7-760 marker, 54 genotypes from entire 169 F progenies showed an expected allele size of 760 bp. These genotypes were selected as candidate for resistance against powdery mildew (Tab. 3). ScORN3-R showed a resistance correlated allele size of 900 bp with both parent ('Regent'-resistance; 'Boğazkere'-susceptible). Therefore, we did not use this marker for MAS to select the candidate resistant genotypes.

All the allelic distributions of SSR markers from 'Regent', 'Boğazkere' and their hybrid plants were scored in Table 2. The number of selected genotypes as candidate for resistance to both mildew diseases and their related resistant loci was performed in Table 3. According to our results, we selected 27 hybrids that carried minimum 2 resistance loci (*Rpv*3 and *Ren*3). 17 hybrids, were selected which contained minimum 2 SSR loci carrying resistance related alleles loci and 1 SCAR locus. 2 hybrids (BR 110 and BR 168) from 169 hybrid plants were selected for further breeding program which harboured all resistance loci (5 SSR loci + 1 SCAR loci) based on the results of our study.

All results were evaluated together with natural inoculation. As a result of natural infection, evaluations were made on the basis of powdery mildew, and BR 168 genotypes were found to be resistant and BR 110 genotype was found to be tolerant (Tab. 4). Our phenotypic results were supporting the data we obtained with the marker-assisted selection. According to this result, molecular studies should be evaluated along with applications of disease infection. Cadle-Davidson et al. [2011] reported that similarly to our work, Run-1 positive hybrids showed different levels of resistance to downy and powdery mildew diseases.

Table 4. Powdery mildew natural infection scores of individuals in the hybrid population

Powdery mildew disease level of hybrids				
1	3	5	7	9
25 BR	18 BR	162 BR	104 BR	122 BR
319 BR	299 BR	30 BR	10 BR	73 BR
23 BR	225 BR	77 BR	237 BR	55 BR
39 BR	14 BR	154 BR	13 KR	206 BR
207 BR	341 BR	19 BR	160 BR	36 BR
87 BR	245 BR	75 BR	45 BR	54 BR
168 BR	57 BR	20 BR	91 BR	142 BR
78 BR	9 BR	63 BR	5 KR	43 BR
21 BR	42 BR	142 BR	159 BR	334 BR
153 BR	292 BR	22 BR	52 BR	335 BR
233 BR	51 BR	235 BR	312 BR	17 KR
107 BR	49 BR	261 BR	145 BR	72 BR
7 KR	125 BR	11 KR	88 BR	296 BR
26 BR	205 BR	113 BR	26 KR	243 BR
13 BR	339 BR	344 BR	343 BR	74 BR
101 BR	154 BR	62 BR	79 BR	99 BR
	144 BR	262 BR	61 BR	27 BR
	243 BR	320 BR	96 BR	258 BR
		110 BR	103 BR	76 BR
			325 BR	3 KR
			137 BR	247 BR
			17 BR	97 BR
			29 BR	278 BR
			32 BR	71 BR
			93 BR	65 BR
			1 KR	146 BR

Marker-assisted selection (MAS) was carried out to follow the inheritance of different loci that originate from different genetic resources of *Vitis* genus. The *Rpv*3 and *Ren*3 loci were introgressed from 'Regent'. Our data also corroborated earlier findings that the 'Regent' derived *Rpv*3 and *Ren*3 loci are closely linked. All markers associated with mildew resistance genes are present in Table 3. Using only the vines that carried both resistance gene of *Rpv*3 and *Ren*3 for further breeding work it can be expected that the proportion of resistant individuals in the progeny will be higher and hence the breeding efficiency will increase.

CONCLUSIONS

Biotechnological techniques and molecular tools have contributed to a better understanding of grapevine genetics and made a great impact on breeding of grapevine. For this reason, these techniques have begun to be used in our country in recent years. First time in Turkey, comprehensive marker-assisted selection method (MAS) in terms of both diseases is made by this study. In addition this study will help to achieve higher frequencies of resistant individuals in future breeding programs. It is planned that the resistance genotypes obtained

by this study will be used in later breeding programs and the resistance studies will be accelerated.

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