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ORIGINAL RESEARCH ARTICLE

Investigation of powdery & downy mildew segregation in Bogazkere hybrids of Turkish wine grape

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ABSTRACT

Powdery mildew (*Erysiphe necator* syn. *Uncinula necator*) and downy mildew (*Plasmapora viticola*) are the most harmful diseases to grapevine production in Turkey. The aim of this study was to generate hybrids between the susceptible Turkish wine grape variety 'Bogazkere' and powdery and downy mildew resistant microvine breeding lines and evaluate their resistance to these important diseases. Marker Assisted Selection (MAS) was used to determine the inheritance of powdery and downy mildew resistance and flower sex loci of the 36 F1 progeny plants obtained by cross-pollination at genotypic level. Based on the PCR results, phenotyping and leaf bioassay were performed to validate initial MAS for 11 and 9 representative progeny with four resistant and susceptible controls. As a result, two tall powdery and downy mildew-resistant vines, three powdery mildew-resistant microvines and two downy mildew-resistant microvines were obtained. The MAS results were also validated by leaf bioassays, which confirmed that resistance genes segregated to the F1 progeny are functional and provide resistance. To the best of our knowledge, this research provides the first photographic validation of the powdery and downy mildew resistance of the Turkish variety 'Bogazkere'. Our findings could potentially provide the wine industry with a resistant wine grape cultivar and an addition to the wine grape germplasm, which could contribute to future breeding programs. Most importantly, the success of the resistance genes segregated into the candidate progeny could help significantly reduce the use of chemicals against grapevine pathogens.

KEYWORDS: *Plasmopora viticola*, *Erysiphe necator*, Bogazkere, *Vitis vinifera*, *Ren4*, *Run1*, *Rpv1*

INTRODUCTION

Grapevines have been cultivated in the region of Caucasia since 6000 BC – 4000 BC and are now cultivated all over the world. (Iland, 2011). Bogazkere is a traditional red berry cultivar grown primarily in Diyarbakır, Turkey. This variety produces balanced wines with fruity notes of strawberry, cherry and blackberry (Ozdemir, 2018).

Erysiphe necator is an obligate biotrophic ascomycete that can infect all the green vegetative tissues of grapevine, generating a unique, white-grey, powdery-like substance. This is a consequence of the existence of hyphae-generating conidiophores and conidia on the surface of the host tissue. The infected grape berries crack open because epidermis growth stops, but the flesh continues to expand. These berry infections cause a decline in yield, grape and wine quality (Evans, 1996). Despite being the main cause of economic losses, chemical spraying is critical for effective management, since winemakers can discard the harvested crop if the powdery mildew level reaches 3 % (Magarey, 2010b). Whilst many *V. vinifera* varieties are highly susceptible to powdery mildew, numerous wild grapevine species indigenous to North America and China show resistance to it (Qiu *et al.*, 2015; Creasy and Creasy, 2018). Growers around the world are heavily dependent upon chemical management strategies, and the use of sulphur-based fungicides are still very common (Magarey, 2010b).

Downy mildew (*Plasmopora viticola*) originates from North America and is one of the most prominent diseases affecting grapevines (Gessler *et al.*, 2011). Long sporangiophores which originate from the stomata cause the downy symptom of the mildew (Spencer-Phillips *et al.*, 2002). Overwintering spores of *P. viticola*, known as oospores, germinate in spring to produce sporangia. Downy mildew affects all the green tissue of the grapevine and can cause a decline in yield if control measures are insufficient (Magarey, 2010a). The majority of cultivated *V. vinifera* varieties are susceptible to downy mildew and its management involves frequent fungicide applications. Foliar spraying is the most common method of downy mildew management, and because multiple pathogen populations grow in an advancing epidemic, multiple chemical spraying within one season is routinely needed to defend plants effectively (Spencer-Phillips *et al.*, 2002).

Throughout their evolution, vascular plants have improved their genetics and to ensure their earned a place within the planet's natural environments (Orton, 2020) Organisms can regenerate themselves via preserved prosperous genes or gene combinations which ensure that new genetic flexibility can emerge continuously (Orton, 2020). Developing resistant grapevines can be considered the key to defending plants from disease. However, it can take up to 15 years to transfer resistant loci to an existing grapevine using conventional breeding approaches (Koledenkova *et al.*, 2022). Prior to beginning a breeding task, the breeding objectives are defined based on, for instance, producer and consumer needs and preferences, and the ecological effects; for example, the aim may be to develop plants which are resistant to insects so that farmers do not need to spray chemicals, or to minimise the amounts they spray (Acquaah, 2012). Both of the current major fungal pathogens of cultivated grapevines, grapevine powdery mildew and downy mildew, were imported into Europe from North America in the second half of the 1800s. As a result, *V. vinifera* has no genetic resistance to either of these two pathogens, meaning that grape growers have to rely on repeated applications of chemicals to reduce their potentially devastating effect on grapevine yield and quality (Dry and Thomas, 2015). The infection of grapevine by *P. viticola* represents the majority of downy mildew infections globally (Lebeda *et al.*, 2008). The annual cost to the Australian grapevine industry of management and yield/quality losses due to powdery and downy mildew pathogen infection can be as high as A$76 and A$63 million respectively (Scholefield and Morison, 2010). Similarly, the grapevine industry in Turkey has lost approximately US$100 million to management and yield losses according to 2018 figures (Prof. Dr. Saadettin Baloglu, personal communication). Chemical spray methods are not only costly, but they are also a health and environmental burden that can impact the marketing image of wines and encourage the occurrence of fungicide resistant pathogens (Merdinoglu *et al.*, 2018). Furthermore, fungicides can impact the health of helpful insects in the vineyard and vineyard staff, as well as enhance carbon emissions due to their repeated application. It would be possible to reduce or entirely solve most of these problems by developing grapevine cultivars with improved genetic resistance to powdery and downy mildew pathogens (Dry and Thomas, 2015).

Various genetic factors that contribute to the resistance of grapevine to downy or powdery mildew have previously been acknowledged (Merdinoglu *et al.*, 2018). Most of them provide varying degrees of resistance to powdery and downy mildew, but a few of them, derived from *V. rotundifolia* and *V. piasezkii*, provide complete resistance genetically (Moroldo *et al.*, 2008). Whether providing partial or complete resistance, many genetic aspects that have been discovered are in genomic sections abundant in Nucleotide Binding Site–Leucine-Rich Repeat (NBS-LRR) resistance genes, known as R genes; the cloning of *Rpv1* and *Run1* has demonstrated that they are associated with this group (Feechan *et al.*, 2013). The French breeder, Alain Bouquet, demonstrated that *Muscadinia rotundifolia* cv. G52 contains a single genetic locus which confers high resistance to both grapevine downy and powdery mildew. This locus was named the *Run1* (Resistance to *Uncinula necator* 1) / *Rpv1* (Resistance to *Plasmopora viticola* 1) locus (Pauquet *et al.*, 2001; Merdinoglu *et al.*, 2003; Dry and Thomas, 2015). Merdinoglu *et al.* (2018) performed MAS for three series of crosses, including Regent, Bronner and Divico, with the aim of generating powdery and downy mildew-resistant varieties containing a combination of *Rpv1*, *Rpv3*, *Rpv10* and *Run1*, *Ren3*, *Ren3.2*. Research in numerous laboratories has indicated that the release of pathogen signalling molecules, named elicitors, which are perceived by plants holding complementary genes, induces plants to start a

hypersensitive response (Keen and Dawson, 1992). Once the infection occurs it stimulates resistance via a number of different defence mechanisms. In fact, the accumulation of phytoalexins produced by the plant, and phenolics and lignin deposition, have been found to enhance the activity of pathogen-related proteins and invoke a hypersensitive response (HR) which reduces pathogen development (Trouvelot *et al.,* 2008). This approach is very useful, as resistance can be provided in plants by the presence of a single dominant R gene (Yoneyama and Hiroyuki, 1993). Nevertheless, the key restriction of conventional breeding for disease resistance is that the end product hybrid also shows unwanted characteristics inherited from the resistant parent and needs multiple backcrosses to remove these characteristics (Merdinoglu *et al.*, 2018). Resistance gene breakdowns have been detected in numerous plant-pathogen interactions. Feechan *et al.* (2013) and Feechan *et al.* (2015) isolated the gene *Run1* from *V. rotundifolia,* which provides complete resistance to powdery mildew. Naturally occuring powdery mildew isolate, Musc4, isolated in North America, is capable of growing on grapevines and avoided *Run1* detection whilst carrying the *Run1* resistance gene (Merdinoglu *et al.*, 2018). Johnson (1979) mentioned that resistance is considered to be strong when its efficiency in a cultivar lasts throughout its commercial period.

Marker assisted selection (MAS) can be defined as being the selection for an attribute according to genotype using relevant markers, rather than according to phenotype. Affordability, reliability, and faster screening are the main reasons for choosing MAS over other methods (Boopathi, 2020). It was thought to be improbable for most of the polymorphisms discovered by the new research methods to be the Quantitative Trait Loci (QTL). (Ben-Ari and Lavi, 2012). The first investigation to evaluate the ease of application of MAS in *V. vinifera* was conducted by Dalbò *et al.* (2001). As MAS can be performed at an early stage of plant development, it has the potential to reduce the number of plants evaluated by the breeder, therefore decreasing expenditure (Ben-Ari and Lavi, 2012). MAS has the major potential to detect effective gene pyramiding, such as the combination of numerous genes in a single variety. With MAS it is possible to generate varieties with very high or absolute resistance by uniting various resistance loci (Merdinoglu *et al.*, 2018). It should be noted that the "quality" and number of markers contribute highly to the success of MAS. The quality of markers is linked to their traits and the cost and effectiveness of genotyping. However, MAS does not reduce the duration of breeding studies, because the candidate individuals still need to be tried and scored in the field. (Ben-Ari and Lavi, 2012).

Up until now, single major genes have been mapped in grapevine for the characteristics of powdery and downy mildew, flower sex and fruit colour, etc. Bouquet (1986) confirmed that complete resistance to powdery mildew originated from *M. rotundifolia* and could be managed by a single dominant locus. This locus, *Run1*, was integrated into the *V. vinifera* genome using a pseudo-introgression method that aimed to obtain new good quality and powdery mildew-resistant grapevine varieties. Pauquet *et al.* (2001) and Donald *et al.* (2002) used a Bulked Segregant Analysis (BSA) approach to identify a number of genetic markers linked to the *Run1* locus and to create a local map near the gene. Barker *et al.* (2005), conducted further studies on physical mapping of the *Run1* gene and constructed a Bacterial Artificial Chromosome (BAC) database from a resistant *V. vinifera* individual containing *Run1* resulting from a backcross. This allowed 20 new genetic markers closely connected to *Run1* to be documented, and the location of the locus to be refined, mapping the gene between SSR marker VMC4F3.1 and the BAC end sequence-derived marker CB292.294 on LG12. Microscopically, *Run1* linked HR, occurring slowly, but it could not prevent hyphal development (Hoffmann *et al.*, 2008). QTLs, such as Ren2 and Ren3 discovered in North America, have been found to be involved in stable and partial resistance. However, unreliable fruit quality, large populations and the many years required for evaluation are some problems linked to interspecific hybrids. *Ren4*, derived from *Vitis romanetii*, is dominant and confers almost complete non-race-specific resistance (Ramming *et al.*, 2011). To date, 27 major QTLs have been discovered in wild *Vitis* species which confer downy mildew resistance (Merdinoglu *et al.*, 2018). However, only Rpv1 from *M. rotundifolia* has been characterised and cloned functionally (Feechan *et al.*, 2013). Regent is a variety from Germany that shows complex resistance to powdery and downy mildew. Derived from *M. rotundifolia*, Regent contains the *Run1* gene which provides resistance to powdery mildew. It also contains Rpv1, which provides resistance to downy mildew (Eibach *et al.*, 2007). Merdinoglu *et al.* (2003) found a close link between Rpv1 and the dominant gene providing resistance to powdery mildew, *Run1*, on LG12. Rpv_{rom} is a downy mildew resistance locus that is linked to the *Ren4* powdery mildew locus in *V. romanetii*; i.e., they are inherited together at a high frequency (Dry *et al.*, unpublished data). However, in grapevines of different origins, Rpv loci have been found to show a broad range of resistance responses (Cadle-Davidson, 2008). This research aimed to assess the degree of resistance/susceptibility of the Bogazkere hybrids of cross-pollinated Turkish wine grape both phenotypically and genotypically. Genotypical assessment based on marker assisted selection and phenotypic assessment was carried out to validate the outcomes of resistance gene conferral.

MATERIALS AND METHODS

1. Pollination

Pollen material of *V. vinifera* cv. 'Bogazkere' was collected in November 2019 from Tallis vineyard in Dookie Victoria (36°21'11.4"S 145°43'26.3"E) to perform the cross pollinations. Between the dates of 12 November 2019 and 4 December 2019, 54 pollinations in total were performed on 10 female microvines carrying *Run1*/*Rpv1* and *Ren4*/Rpv_{rom} resistance genes. The physiologically mature fruit was collected in March 2019 and the seeds extracted prior to embryo rescue.

2. Embryo rescue & tissue culture

The modified embryo rescue protocol of Chatbanyong and Torregrosa (2015) was applied to the seeds generated after pollination (see Table S1). Once the seeds had been extracted, a modified version of tissue culture protocol was performed based on Torregrosa *et al.* (2001). The seed was divided into four groups depending on their parents (Cross 2, Cross 3, Cross 5 and Cross 6) and codes were given to the progeny (e.g., code 6.1 indicates Cross 6 – Plant 1). Seed germination and seed sterilisation were performed using the following protocol. The seeds were left on a shaker overnight at 100 rpm in 0.5M H_2O_2. They were then rinsed in sterile water and transferred to tubes to which 2.6 mM Gibberellic acid potassium salt (GA3) was added, and they were then left to shake overnight at 100 rpm. The seeds were then washed in sterile water and transferred to a 50 mL Greiner™ tube plugged with absorbent cotton wool to approximately the 40 mL mark, which had been wetted carefully with nanopure water and autoclaved. The seeds were layered on top and the tube was sealed with Parafilm™. For the germination, four pieces of 90 mm Whatman No1. Filter Paper™ were layered in 100 x 15 mm Petri dishes. The filter paper was wet with 5.6 mL sterile water. The seeds were then placed on top (20 seeds per plate), and the lid was replaced and the petri dish was sealed with four layers of Parafilm™. The plates were covered with foil and stored in the dark until germination had finished. Once they had germinated, the plants were transferred to 70 mL rooting media (R.M) (see Table S2) and stored in a growth room prior to DNA extraction.

3. DNA extraction

A modified version of the DNA extraction protocol of Zhang *et al.* (1998) was used to obtain genomic DNA. A leaf was ground to fine powder in 800 µL of Cetyl Trimethylammonium Bromide (CTAB) buffer and 1.6 µL ß-mercaptan was added; this mixture was incubated at 60 °C for 20 min. Next, six hundred µL of chloroform isoamyl alcohol (24:1) was added, and the mixture was vortexed and centrifuged at 13.000 rpm for 5 min. The supernatant (500 µL) was transferred to a new tube, and an equal amount of isopropanol was added. The tube was then placed on ice for 10 min to precipitate the DNA. The DNA was pelleted by centrifuge at 13.000 rpm for 15 min, 200 µL of 70 % ethanol was then added to rinse the pellet, and it was centrifuged again at 13.000 rpm for 5 min, after which the ethanol waste was removed. The DNA pellet was dried and resuspended in 50 µL H_2O. Extracted DNAs was tested via Thermo Scientific NanoDrop™ spectrophotometer and elongation factor PCRs.

4. MAS

MAS was performed to investigate the segregation of desired traits in 36 candidate F1 progeny plants (See Table S4 for PCR preparation chart). All the markers were primarily tested with controls. and *Ren4*/*Rpv*$_{rom}$ and the flower sex marker (VVMT137) were identified using CAPS markers, which involved PCR followed by a restriction digest. The *Run1*/*Rpv1* locus was identified with a dominant marker and only the PCR product was used. PCR *Ren4*/*Rpv*$_{rom}$ in 1.2 %, *Run1*/*Rpv1* in 1.2 % and flower sex marker (VVMT137) were amplified in 2 % agarose gel with a 100-watt electric current. An Owl™ EasyCast™ B2 Mini Gel tank was used for this step.

5. Pathogen material

Powdery mildew pathogen was obtained from isolated infected Cabernet-Sauvignon grapevine leaves. Downy mildew pathogen was obtained from infected discs, which were prepared weekly. Both of the pathogens used for the inoculations were stored in controlled conditions.

6. Phenotyping & leaf bioassays

Inoculations and scoring for powdery and downy mildew infections were performed at least twice on four controls, and 11 and 9 representative progeny. Powdery and downy mildew infections was visually assessed using the following scale: 0 (absence of infection), + (weak infection), ++ (medium infection), +++ (strong infection) (see Tables 1 and 2). Photographs of powdery and downy mildew infected leaf tissue were taken with a Nikon SMZ25 Stereomicroscope™ (Japan).

Downy mildew leaf disc inoculations were performed according to Feechan *et al.* (2013). The downy mildew's total sporangia per disc was counted for at least two replicates of the controls and 9 representative progeny. A leaf disc assay was performed using the following protocol. All the materials were first sterilised using 70 % ethanol. Five mL of water and previously infected discs were mixed to produce a sporangial suspension for inoculation. The concentration of sporangia in the suspension was measured with a Fucsh-Rosenthal counter and was adjusted to 5 x 10^4 to achieve sporangia per mL. One 5th of the leaves on the stem of each progeny plant was collected and gently washed in deionised water. Five discs per leaf (1.5 cm diameter) were punched out and placed upside down on wet filter paper. Once the disc surface was dry, a 20 µL drop was placed in the middle of the disc and the container was wrapped in cling wrap to maintain high humidity and then stored in an incubator at 22-24 °C with 16 hours of light and 8 hours of darkness. The following day, the drops on the discs were dried and the tray was re-wrapped. The discs were scored for powdery and downy mildew infection after 6 days.

Powdery mildew inoculations were performed at least twice on 11 representative progeny and four controls according to a modified version of Donald *et al.* (2002). The collected leaves were labelled, washed in Milton™ solution (Tween added, 100 µL/L) for 3 min and swirled often in test jug. They were then rinsed with sterile water and each leaf was placed on a plastic stick on the surface of agar in a Petri dish and left to dry. Once dry, each leaf was placed on the highly infected leaf and gently inoculated using a paintbrush by surface contact. Lids were placed on the Petri dishes, which were then sealed with 3M™ tape and stored in the 24 °C incubator. The leaves were scored for powdery and downy mildew infection after 6 days.

RESULTS

1. Marker validation

Prior to performing Polymerase Chain Reactions (PCRs) on the F1 segregating progeny, the parent Bogazkere DNA extracted from rachis tissue was tested with *Run1*/*Rpv1*, *Ren4*/*Rpv*$_{rom}$ and flower sex markers to validate the compatibility between the variety and markers. The *Run1*/*Rpv1* marker is dominant and is based on the absence or presence of a DNA amplified product. The *Ren4*/*Rpv*$_{rom}$ and flower sex markers are co-dominant CAPS markers which require a restriction enzyme to cleave the PCR product in order to indicate any differences in fragment sequence and to create multiple bands. The *Run1*/*Rpv1* marker used in this study was a dominant marker that has a band of 302 bp when the locus is present in the DNA and is blank when the gene is absent. According to the results shown in Figure 1, parent Bogazkere does not carry the *Run1*/*Rpv1* locus.

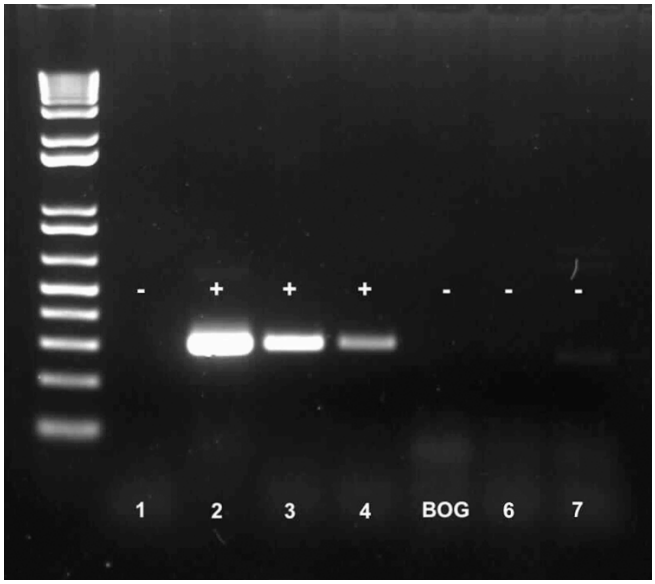


FIGURE 1. PCR results of *Run1/Rpv1* resistance marker test (Lane 1 is negative control H_2O, lanes 6 and 7 are negative control varieties absent of *Run1/Rpv1* respectively. Lanes 2, 3 and 4 are positive control varieties carrying *Run1/Rpv1*, BOG is parent Bogazkere, - and + indicate absence and presence of *Run1/Rpv1* respectively).

The *Ren4*/*Rpv*$_{rom}$ marker used in this study is a co-dominant CAPS marker which is informative and reliable and requires an *ApoI* restriction enzyme to differentiate the PCR products. Three DNA bands indicate resistant individuals and one band indicates susceptible individuals. According to the results shown in Figure 2, the parent Bogazkere did not show a *Ren4*/*Rpv*$_{rom}$ locus.

The VVMT137 flower sex marker used in this study is a co-dominant CAPS marker that requires the *RsaI* restriction enzyme to differentiate the PCR products. The female individuals show as a single band, whereas hermaphrodite DNA shows as three bands. According to the results shown in Figure 3, the parent Bogazkere showed hermaphrodite sex.

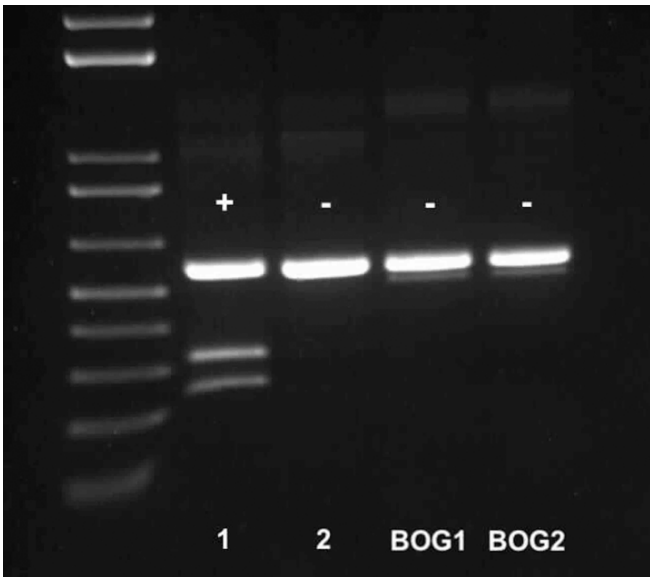


FIGURE 2. PCR results of *Ren4/Rpv*$_{rom}$ resistance marker test (Lane 1 is a positive control variety carrying *Ren4/Rpv*$_{rom}$, Lane 2 is a negative control variety absent of *Ren4/Rpv*$_{rom}$ and BOG1-BOG2 are parent Bogazkeres, - and + indicate absence and presence of *Ren4/Rpv*$_{rom}$, respectively).

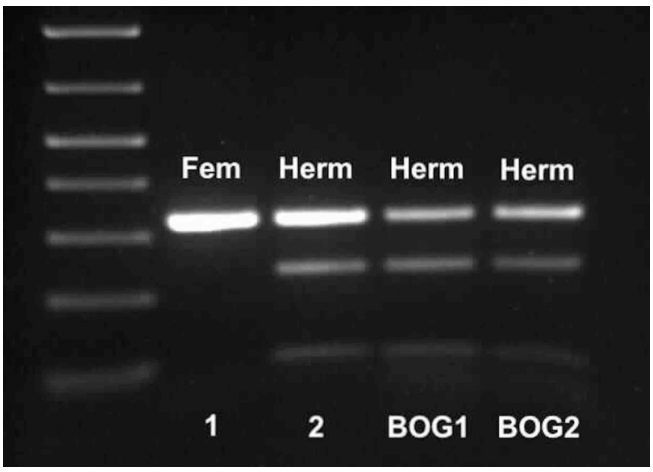


FIGURE 3. PCR results of VVMT137 flower sex marker test (Lane 1 is a control variety for female sex, Lane 2 is a control variety for hermaphrodite sex, BOG1-BOG2 are parent Bogazkeres, Fem and Herm indicate flower sex respectively).

2. Genetic evaluation of segregated progeny

DNA extractions were performed on 36 F1 progeny, and the segregation of the traits of the parents were assessed via PCR analysis following the successful validation of *Run1*/*Rpv1* and *Ren4*/*Rpv*$_{rom}$ resistance and VVMT137 flower sex markers.

The dominant *Run1*/*Rpv1*marker has one band if the locus is present and no band if the locus is absent.

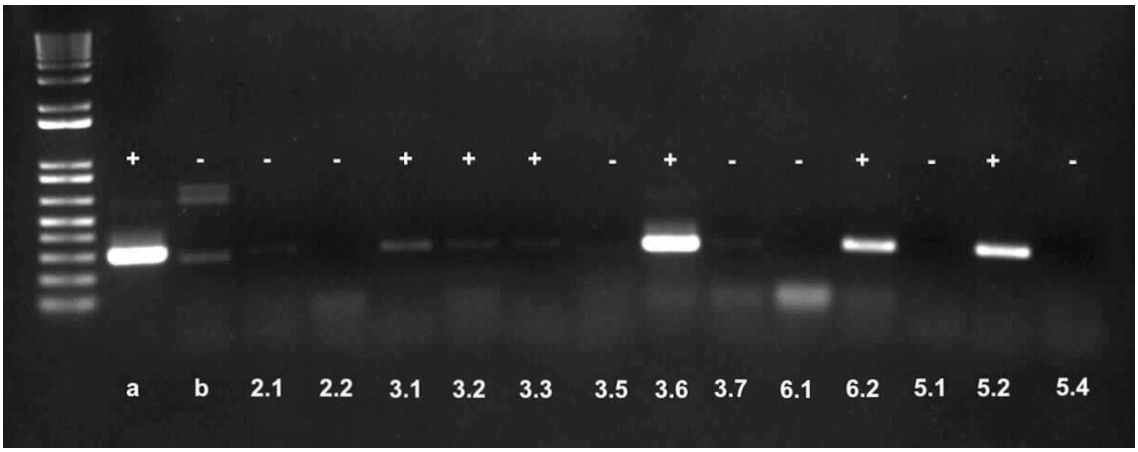


FIGURE 4. Representative results of *Run1/Rpv1* resistance marker of Bogazkere F1 progeny (Lane a is a positive control carrying *Run1/Rpv1*, Lane b is a negative control absent of *Run1/Rpv1*, - and + indicate absence and presence of *Run1/Rpv1* respectively) (see Table S6, Figure S1).

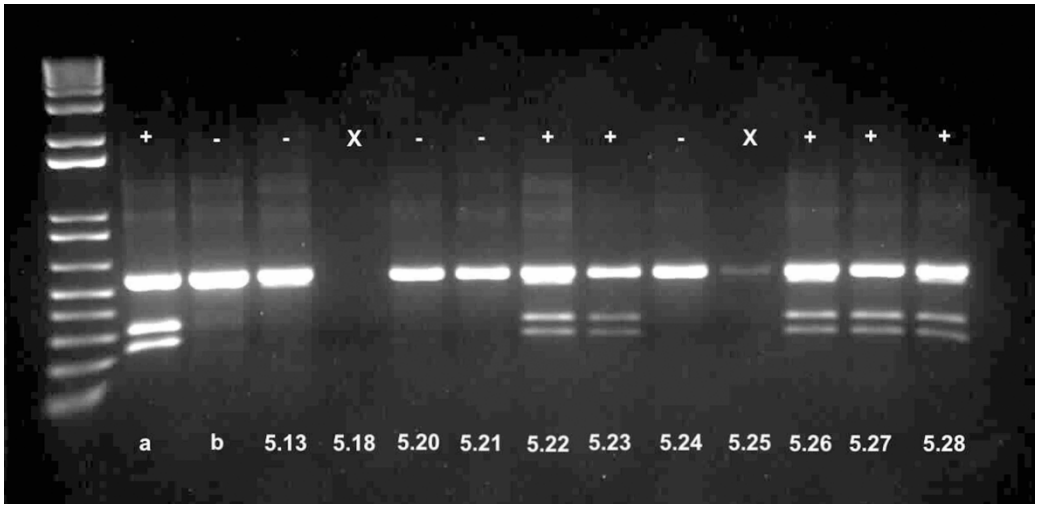


FIGURE 5. Representative results of *Ren4/Rpv*$_{rom}$ resistance marker of Bogazkere F1 progeny (Lane a is a positive control carrying *Ren4/Rpv*$_{rom}$, Lane b is a negative control absent of *Ren4/Rpv*$_{rom}$, X is n.d., - and + indicate absence and presence of *Ren4/Rpv*$_{rom}$ respectively) (see Table S6 and Figures S2-S3).

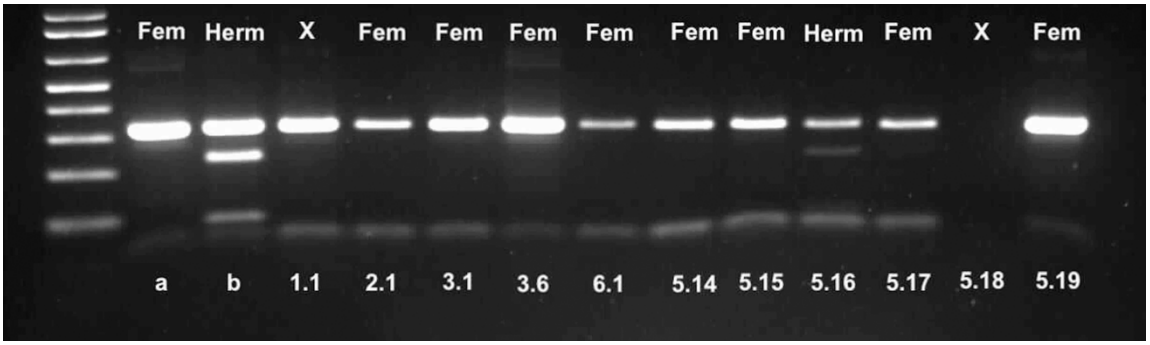


FIGURE 6. Representative results of VVMT137 flower sex marker of Bogazkere F1 progeny (Lane a is a female control for flower sex, Lane b is a hermaphrodite for flower sex, X is n.d., Fem and Herm are flower sex respectively) (see Table S7, Figures S4-S5).

According to the representative results shown in Figure 4, 13 of the 36 progeny were carrying a *Run1*/*Rpv1* locus.

The *Ren4*/*Rpv*$_{rom}$ resistance marker is a co-dominant CAPS marker with three bands if the progeny is resistant or one band if the progeny is susceptible. According to the representative results shown in Figure 5, 21 of the 36 progeny were carrying a *Ren4*/*Rpv*$_{rom}$ locus.

The flower sex marker lane is expected to show one 302 bp band for female progeny and 3 bands for hermaphrodite progeny. According to the representative results shown in Figure 6, 16 of the 36 progeny were female plants and 20 of the 36 progeny were hermaphrodite plants.

The results of the MAS for resistance genes and flower sex led to the selection of 25 progeny lines to be transferred to the glasshouse for phenotyping (see Table S8). The plants were categorised for further evaluation based on their flower sex and resistance gene presence.

3. Phenotyping of segregated progeny

In July 2020, 25 of the 36 progeny were successfully transferred to the glasshouse; nine of 36 progeny plants could not survive the transfer from the tissue culture to the glasshouse. The grape soil mix used to transfer the plants can be seen in Table S3, and plant types, planting dates and glasshouse transfers of Bogazkere F1 progeny are provided in Table S5. Phenotyping was performed in order to (a) determine the inheritance of the dwarfing phenotype from the microvine parent, (b) validate the genotypic and phenotyping performed to validate the genotypic results of *Ren4*/*Rpv*$_{rom}$ and *Run1*/*Rpv1* resistance markers, and (c) confirm that the resistance genes, successfully segregated to F1 hybrids, were genetically functional against both powdery and downy mildew (see Table S6). Phenotyping for plant type was performed in the glasshouse by visual scoring and it was not necessary to use DNA markers to distinguish the trait as shown in Figure 7.



FIGURE 7. Visual scoring of progeny plants in glasshouse. Plant a represents a dwarf progeny and plant b represents a tall progeny.

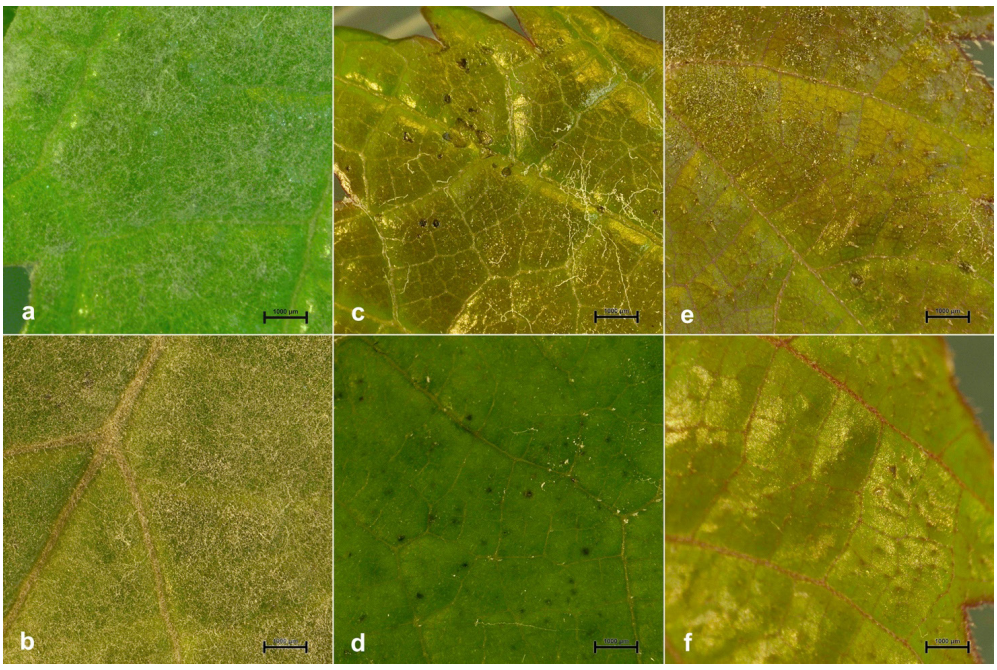


FIGURE 8. Powdery mildew leaf bioassay images of three controls and three representative progeny under 1.5x magnification.

TABLE 1. Detached powdery mildew leaf bioassay scoring chart of 11 representative progeny and 4 controls (Cabernet-Sauvignon and L1 are susceptible controls absent of *Ren4* and *Run1*, VIP and 138-40 are resistant controls carrying *Ren4* and *Run1*, +++ = strong infection, ++ = medium infection, + = weak infection, 0 = no infection; the results show a range of two replicates).

Cross Number	Plant Type	*Ren4*	*Run1*	Hyphae	Sporulation	Hypersensitive Response	Replicate
Cabernet-Sauvignon	Tall	-	-	+++	+++	0	2
L1	Micro	-	-	+++	+++	0	2
VIP	Tall	-	+	0/++	0/+	0/+++	2
138-40	Tall	+	-	0/+	0	+/+++	2
5.6	Micro	-	+	0	0	+++	2
5.20	Micro	-	+	+	0	++/+++	2
5.11	Tall	+	-	+	0	++	2
5.17	Tall	+	-	0/+	0	+++	2
5.7	Micro	+	-	+/++	0	+/++	2
3.2	Tall	+	+	0	0	+++	2
3.3	Tall	+	+	0	0	+++	2
5.9	Micro	+	+	0/+	0/+	+++	2
5.24	Micro	-	-	++	++	0	2
5.21	Tall	-	-	+++	+++	0	2
5.16	Tall	-	-	+++	+++	0	2

TABLE 2. Downy mildew leaf disc bioassay phenotyping data for 9 representative progeny and 4 controls (Cabernet-Sauvignon and L1 are susceptible controls absent of *Rpv1* and Rpv_{rom}, VIP and 138-40 are resistant controls carrying Rpv_{rom} and *Rpv1*, +++ = strong infection, ++ = medium infection, + = weak infection, 0 = no infection; the results show a range of two to three replicates, 5.6 and 5.20 are n.d.- not determined).

Cross No.	Plant Type	Rpv_{rom}	*Rpv1*	Sporulation	Hypersensitive Response	Replicate
Cabernet-Sauvignon	Tall	-	-	+++	0	3
L1	Micro	-	-	+++	0	3
VIP	Tall	-	+	+/+++	0/+++	3
138-40	Tall	+	-	0/+	+++	3
5.11	Tall	+	-	0/+	++	3
5.17	Tall	+	-	0/++	0/+++	3
5.7	Micro	+	-	+/++	++/+++	3
3.2	Tall	+	+	0/+	+++	3
3.3	Tall	+	+	0/+	+++	3
5.9	Micro	+	+	0/++	++/+++	3
5.24	Micro	-	-	+++	0	3
5.21	Tall	-	-	+++	0	2
5.16	Tall	-	-	+++	0	2

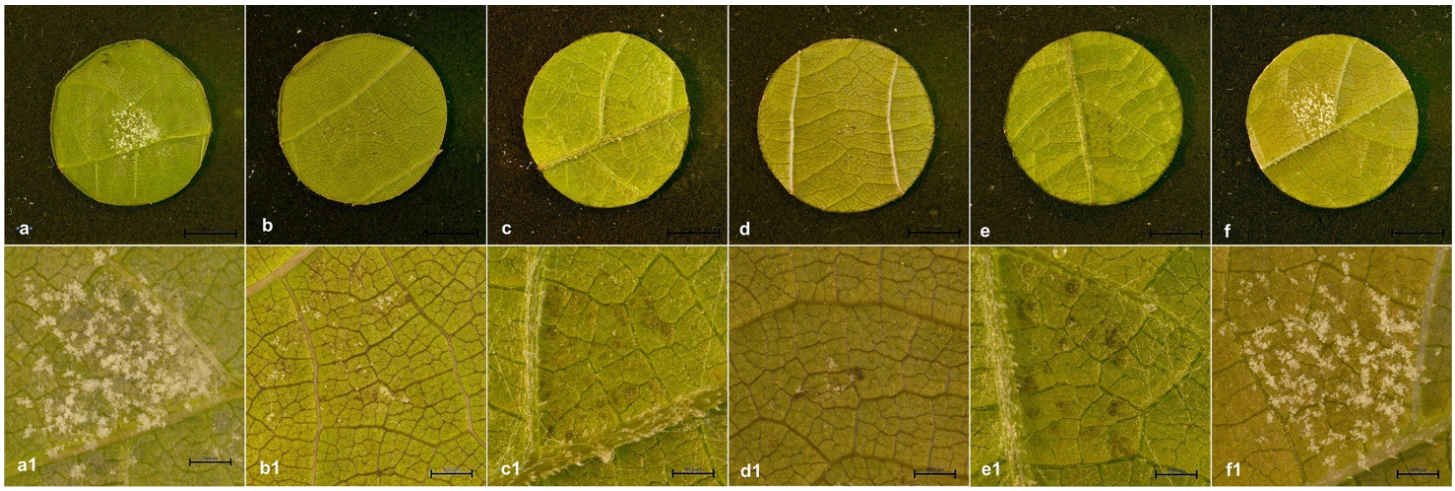


FIGURE 9. Downy mildew leaf disc bioassay images of three controls and three representative progeny under 0.5x and 2x magnification respectively.

Powdery mildew resistance/susceptibility was assessed by carrying out a detached leaf assay. As shown in Figure 8, the susceptible control genotype Cabernet-Sauvignon (panel a) and progeny line (panel b) show strong hyphal growth, sporulation and no HR. In contrast, the resistant control 138-40 (panel c) and the progeny line 5.17 (panel d), carrying the *Ren4* gene, show minimal hyphal growth, sporulation and strong HR. Progeny line 3.3 (panel e), carrying both *Ren4* and *Run1,* showed very strong HR and no hyphae or sporulation.

The results of the powdery mildew detached leaf bioassay show a correlation between the resistant controls and the resistant progeny lines and the susceptible controls and the susceptible progeny lines. Figure 8 shows: a) Cabernet-Sauvignon, b) 3.7, c) VIP, d) 138-40, e) 5.17, and f) 3.3, where a is a susceptible control, b is a susceptible progeny, c and d are resistant controls carrying *Run1* and *Ren4* respectively, e is a resistant progeny carrying *Run1*, and f is a resistant progeny carrying both *Ren4* and *Run1*. The powdery mildew images shown in Figure 8 also validate the phenotypic results of the representative progeny groups shown in Table 1.

Downy mildew resistance/susceptibility was assessed in a leaf disc assay. In Figure 9, the susceptible controls Cabernet-Sauvignon (panel a), microvine L1 (panel b) and progeny line 3.7 (panel h) show dense sporulation and no HR. The resistant control 138-40 (panel d) and progeny line 5.17 (panel f), which carry the Rpv_{rom} resistance gene showed minimal sporulation and strong HR. The resistant control VIP (panel c), carrying the *Rpv1* resistance gene showed minimal sporulation and strong HR. Progeny line 3.3 (panel g) carrying both *Ren4* and *Run1* showed very strong HR and no sporulation.

Figure 9. a. Cabernet-Sauvignon, b. VIP, c. 138-40, d. 5.17, e. 3.3, f. 3.7, where a is a susceptible control absent of *Rpv1* and Rpv_{rom}, b and c are resistant controls carrying *Rpv1* and Rpv_{rom} respectively. d and e are resistant progeny carrying *Rpv1*, Rpv_{rom} and both *Rpv1* and Rpv_{rom}, respectively and f is susceptible progeny absent of *Rpv1* and Rpv_{rom}, a1, b1, c1, d1, e1 and f1 are the 2x magnified versions of a, b, c, d, e and f.

The downy mildew leaf disc bioassay gave similar results with resistant controls and resistant progeny lines along with susceptible controls and susceptible progeny lines, and validated the phenotyping results shown in Table 2.

The downy mildew sporangia/disc for four controls and 9 representative progeny showed results parallel to the genotypic and phenotypic results. Progeny line 5.6 and 5.20 could not be determined due to non-mature progeny in the downy mildew leaf bioassay. Cabernet-Sauvignon, L1, VIP, 138-40, 5.11, 5.17, 5.7, 3.2, 3.3, 5.9, 5.24 were replicated three times, and 5.21 and 5.16 were replicated twice as shown in Table 2.

DISCUSSION

Powdery and downy mildew are known to be major diseases of grapevine (Campbell, 2004). For reasons such as economic losses and health-environment problems due to extensive chemical applications, the importance of resistance breeding research for the grape industry is increasing (Dry and Thomas, 2015; Scholefield and Morison, 2010). The present study aimed to obtain powdery and downy mildew-resistant hybrids of the Turkish wine grape 'Bogazkere'. Resistant female microvines were pollinated with Bogazkere pollen, their seeds were extracted, the embryo rescued, and the germinated seeds transferred to tissue culture. Once the progeny plants were mature enough, DNA was extracted and MAS performed to determine segregated resistance and flower sex for candidate progeny via *Ren4*/Rpv_{rom}-*Run1*/*Rpv1* markers and a VVMT137 marker respectively. Following the MAS, the plants were transferred to the glasshouse for further phenotyping and the study was continued on 25 progeny out of a total of 36. In a similar study, Vezzulli *et al.* (2019) investigated 100 grapevine progeny lines in-vivo for powdery and downy mildew resistance; interestingly, the results showed a lower number of powdery mildew resistant progeny lines compared to downy mildew resistant progeny lines. This could be due to varietal differences conferred from the parents.

These results highlight the importance of making additions to genetic resources (Vezzulli *et al*., 2019).

In the next part of the present study, phenotyping based on the MAS results was carried out and powdery and downy mildew resistance was investigated on 11 and 9 representative progeny respectively, and four controls in four groups based on their resistance gene presence or absence (Table 1 and 2). However, in the *Run1* group, only two progenies, 5.6 and 5.20, were carrying *Run1* out of the whole population and were evaluated for powdery mildew. Progeny 3.2 and 3.3 carryied both *Ren4* and *Run1* genes, thus indicating strong resistance, with HR and no sporulation. Similarly, 5.9, carryied both *Ren4* and *Run1* genes, indicating resistance, and they showed minimal hyphal development and sporulation. The progenies 5.6 and 5.20 carried the *Run1* gene and 5.11, 5.17 and 5.7 the *Ren4* gene, showing a similar degree of hyphae, sporulation and HR to the resistant controls VIP and 138-40. Susceptible progeny 5.24, 5.21 and 5.16 showed strong hyphal growth, sporulation and no HR, as was expected. Phenotyping for powdery mildew resistance validated the MAS results and indicated similar resistance by both *Ren4* and *Run1*. In their study, Pimentel *et al*. (2021) investigated the defence responses of grapevines prone to powdery mildew via transcriptional, hormonal and metabolic alterations. The results showed that the berries were capable of triggering a defence mechanism. Similar activity was observed in the leaves of Cabernet-Sauvignon. This aligns with the finding that powdery mildew-subjected grapevines are able to initiate resistance in diverse parts of the plants, but this is not effective enough for stopping or counter attacking the powdery mildew (Pimentel *et al*., 2021; Fung *et al*., 2008; Fekete *et al*., 2009).

According to the downy mildew leaf disc assay, progenies 3.2 and 3.3 carried both Rpv_{rom}/*Rpv1*, thus indicating strong resistance, and had a minimal sporulation of 5,000 and 1,000 sporangia/disc respectively, which is similar to that of the resistant control VIP and 138-40, with 3,722 and 667 sporangia/disc respectively. 5.9 carried both Rpv_{rom} and *Rpv1*, indicating a degree of resistance, but it had significantly higher sporangia/disc than 3.2 and 3.3. Progenies 5.11 and 5.17 also showed strong resistance, with 1,333 and 2,917 sporangia/disc respectively. The results of the phenotype test showed that *Rpv1* and *Rpv3* loci significantly increased resistance against downy mildew. Homozygous plants which carry the resistance genes *Rpv1* and *Rpv3* are useful tools for researchers and can be used to generate novel and exclusive varieties (Sánchez-Mora *et al.*, 2017). Similar to microvine 5.9, progeny 5.7 carrying Rpv_{rom}, showed a degree of sporulation and significantly higher sporangia/disc. Susceptible 5.24, 5.21 and 5.16 showed strong sporulation and no HR with very high sporangia/disc counts as expected, which were even higher than the susceptible controls Cabernet-Sauvignon and L1. This can be explained by the number of replicate differences in the group of susceptible progenies 5.16, 5.21 and 5.24. Furthermore, different individuals carrying the same key resistance genes can exhibit different levels of resistance as shown in a statistical analysis conducted by Sánchez-Mora *et al.* (2017). However, in the present study, due to Covid-19, the schedule of the research was negatively affected and the leaves were not mature enough to conduct the experiment using a third replicate, for example progeny lines 5.6 and 5.20.

Phenotypical assessment plays a key role in improving the breeding of members of a population sharing the same environment, given the existence of resistance loci pyramids in their genome (Zini *et al.*, 2019). In this study, phenotyping validated the MAS results, and the segregation and functionality of the resistance genes. The resistant progeny showed clear-cut HR and lack of powdery and downy mildew infection. Bellin *et al.* (2009) found a strong link between the capability of a plant to mount an HR in controlled environments and the quantitative resistance of a mature plant predisposed to natural contagions in the vineyard, as shown by the numerous leaves with sporulation in two repeated seasons of observations. When combined, phenotyping and MAS are a useful tool for providing new genotypes with partial resistance, as well as determining the robustness of the resistance more precisely (Ruiz-García *et al.*, 2021). Furthermore, the use of molecular markers to forecast desired attributes, alongside propagative methods to shrink the time gap between generations, could significantly enhance the genetic gain of plant and animal species (Meuwissen *et al*., 2001).

The microvine progeny 5.7 and 5.9 that carried *Run1*/*Rpv1* and/or *Ren4*/Rpv_{rom} loci have the potential to be parents in future breeding projects. A comparison of physical and genetic mapping data has revealed recombination to be greatly repressed in the area of *Run1*; this is thought to be due to differentiated sequence inside the backcrossed fragment of *M. rotundifolia* which holds the *Run1* loci and this loci is linked to *Rpv1* (Barker *et al.*, 2005; Sánchez-Mora *et al.*, 2017). In the present study, the downy mildew sporangia/disc count of microvines 5.7 and 5.9 were also significantly higher than that of the tall vines which both conferred the same resistance genes and probably require further assays including only microvines. The progeny lines 3.2 and 3.3, which carry both the *Ren4*/*Run1*and *Ren4*/Rpv_{rom} resistance loci with tall plant type and hermaphrodite flower sex, are potential progeny for field evaluation of viticultural characteristics and wine quality in the coming years.

CONCLUSION

This study successfully generated F1 'Bogazkere' hybrids which carry *Run1*/*Rpv1* and/or *Ren4*/Rpv_{rom} resistance loci. The segregation of resistance genes could be adapted to table grape and raisin grape varieties More importantly, the results of this research could benefit the wine industry by helping decrease its carbon footprint and chemical use, as well as contributing to conservation in the environment, beneficial insects and the health of grape producers. The resistant F1 'Bogazkere' wine grape hybrids could be adapted to the Australian and Turkish wine industries: the sensory attributes of the wine could be assessed in small batch winemaking

and one or two backcrosses may be required to increase the 'Bogazkere' percentage of the hybrids, and thus obtain high quality wine grape that is resistant to powdery and downy mildew. The table and raisin grape industries could also benefit from further research on the introduction of resistance genes to other varieties from different *Vitis* subspecies.

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