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Research Article

Marker assisted selection (MAS) for downy mildew resistance in grapevines using Rpv3.1 associated markers

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Abstract

Powdery mildew and downy mildew are primary fungal diseases that cause significant damage in viticulture. Therefore, breeding powdery and/or downy mildew resistance is one of the priority subjects in grapevine breeding programs. This study aims to conduct early-selection by marker assisted selection (MAS) method among 869 genotypes obtained through crossbreeding 'Alphonse Lavallee' × 'Regent' cultivars using the markers (GF18-06 and GF18-08) associated with downy mildew resistance gene region *Rpv3.1* to develop new grapevine cultivars resistant to downy mildew caused by *Plasmopara viticola*. A total of 869 hybrid plants which were obtained after crossing 'Alphonse Lavallee' × 'Regent' in a 3-year breeding program were used in the study. The hybrid plants were scored for the resistance level based on their sporulation intensity after artificial inoculation of *P. viticola*. DNA samples of the hybrid plants were amplified with GF18-06 and GF18-08 markers in Polymerase Chain Reaction (PCR) for MAS. The alleles which were associated to *Rpv3.1* resistance locus and the results of resistance scoring were compared, and the applicability of the markers in MAS was verified. It was determined that the GF18-08/410 bp marker can be used successfully for MAS. Gf 18-06 marker 385 bp, 390 bp and 407 bp gave false positive results in our population, respectively 8.86%, 9.02% and 37.94%. Therefore, this may limit its use for MAS.

Keywords: GF 18-08; GF 18-06; grapevine; marker assisted selection; *Plasmopora viticola*; *Rpv3*

Introduction

Grapevine (*Vitis vinifera* L.) is one of the most important perennial crops with high commercial value in the world. Fungal diseases are among the most important concerns of viticulture and cause high losses in vineyard yields. They may also affect the yield of the following year depending on the severity of the disease during the growing period. It may sometimes dry up all vineyard, which poses a great risk for the producer. Today, many fungicides are used quite successfully against fungal diseases. However, a great amount of fungicide is required to reduce the crop loss due to downy mildew disease in grapevine. More than 15 spraying

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may be necessary per season in years of highly humid climatic conditions. The excessive pesticide uses increases production costs and, pollutes natural resources. The striking results of research on the amounts of fungicide in viticulture clearly present the significance of the studies for limiting the use of chemicals for sustainable viticulture (Wingerter *et al.*, 2021). Merdinoglu *et al.* (2018) state that 33% of all the fungicide in Europe are used in vineyards. Data show that approximately 50% of all the fungicide used in European Union countries are actually being applied in vineyards which make up %5 of total farmland in EU (Zendler *et al.*, 2017). On the other hand, EU directive 2009/128 for sustainability management of diseases caused by plant pathogens in the EU recommends a reduction in the number of fungicide treatments in the field (Sargolzaei *et al.*, 2020).

In contrast to rapid increase in world population, the natural areas in cities are gradually decreasing which has led to an increase in people's need for a healthy life. This is among the reasons why individuals' eating habits are changing. Crops grown using minimal chemicals have become more popular than conventional farming, and consumers have come to prefer them more. Consumer preferences and the problems faced by growers have changed the direction of breeding studies in viticulture as in many agricultural branches and resulted in demands for products of the plants that are especially resistant or tolerant to diseases.

Although some Vitaceae species have developed resistance mechanisms against diseases caused by fungi, they do not seem to possess the level of quality that most consumers demand (Agurto *et al.*, 2017). Therefore, the crossing is the first among the methods which are used to develop new grapevine cultivars with high quality as well as tolerance to disease and pests. However, classical plant breeding programs are long-term applications and they require years of observation on hybrid plants to see the targeted characteristics.

As a result of the inclusion of biotechnological methods in breeding programs, genetic analyzes related to disease resistance in grapes were carried out by different researchers, resistance-related gene regions have been identified. Molecular markers associated with these resistance-related gene regions have been developed (Akkurt *et al.*, 2007; Welter *et al.*, 2007; Hoffmann *et al.*, 2008; Katula-Debreceni *et al.*, 2010; Riaz *et al.*, 2011; Di Gaspero *et al.*, 2012; Zyprian *et al.*, 2016). Thanks to the inclusion of the MAS in breeding programs, breeding processes have been shortened, and hybrid plants that do not have the desired character or characteristics can be identified and removed from breeding programs without the need for phenotypic observations. In this way, land, labour, time, and costs in breeding programs have been greatly lowered.

The gene regions that provide resistance to fungal diseases in grapevine are called Rpv (Resistance to *Plasmopora viticola*) for downy mildew, Run (Resistance to *Uncinula necator*) and Ren (Resistance to *Erysiphe necator*) for powdery mildew. 28 gene regions associated with resistance to downy mildew (*Plasmopara viticola*) and 13 gene regions associated with resistance to powdery mildew (*Erysiphe necator*) in grapevine have been reported so far (Vitis International Variety Catalogue VIVC, 2022). It is also known that the majority of downy mildew resistant cultivars grown in Europe were developed from a single major resistance locus, *Rpv3.1* (Eisenmann *et al.*, 2019).

The cultivar 'Regent', which was bred as resistant to fungal diseases, was developed at "Institute for Grapevine Breeding Geilweilerhof" (Siebeldingen/Germany) in Germany in 1996 (Eibach and Töpfer, 2003). *Rpv3.1* gene region, which is associated with downy mildew resistance, was first identified in the genetic map of the cultivar 'Regent' (Fischer *et al.*, 2004; Welter *et al.*, 2007) and was described in more detail in 'Bianca' (*V. vinifera* cv.) (Di Gaspero *et al.*, 2012). Van Heerden *et al.*, (2014) reported that some Run and Ren genes are located in the same region as *Rpv3.1* gene. The resistance mechanism of 'Regent' to powdery mildew is based on a 'post-contamination' mechanism that restricts pathogen growth and inhibits conidium formation (Zendler *et al.*, 2017). It is known that pathogens that cause fungal diseases in viticulture have different races as well. Therefore, resistance breeding studies aim to combine many gene regions (gene pyramiding) in order to stand against the aforementioned pathogen diversity (Zendler *et al.*, 2017; Zini *et al.*, 2019). It is seen that many recent breeding programs (Eibach *et al.*, 2007; Schwander *et al.*, 2012; Venuti *et al.*, 2013) aim at pyramiding resistance gene regions and transferring these pyramided gene regions to new cultivars (Saifert *et al.*, 2018). Therefore, cultivars such as 'Regent', which possess gene regions in their genomes associated with

resistance to different diseases and high-quality characteristics, have high significance and value for breeding studies as well as growers.

This study aims to conduct early-selection via marker assisted selection (MAS) among 869 genotypes obtained through crossing 'Alphonse Lavallee' × 'Regent' cultivars using the markers (GF18-06 and GF18-08) associated with resistance gene region *Rpv3.1* to breed new grapevine cultivars resistant to downy mildew. Additionally, hybrid genotypes were scored based on disease severity after being infected with artificial inoculation. By comparing the markers associated with the *Rpv3.1* gene region (GF18-06; GF18-08) with the resistance levels, it was aimed to confirm the usability of these markers for MAS purposes in grape breeding studies for resistance to downy mildew disease.

Materials and Methods

Plant material

Eight hundred sixty-nine offspring from the crossing population of 'Alphonse Lavalleé' (susceptible *V. vinifera* cv) x 'Regent' (Interspecific hybrid, complex resistance against downy and powdery mildew) were used in this study.

DNA isolation and PCR amplification

Young leaf samples (0.5-1 g) were collected from each plant and kept at -80 °C until use. DNA isolations were performed according to the Plant/Fungi DNA Isolation Kit (Norgen Biotek Corp.) protocol using healthy young leaf samples. Genomic DNAs from the samples were run at 100 V for 1 hour with Agarose Gel Electrophoresis method and visualized by SynGene imaging system. The amount and purity of the DNA were measured with the Nano Drop ND-1000 Spectrophotometer. The final concentration was adjusted to 10 ng ul^{-1}.

Two SSR markers, GF 18-06 and GF 18-08, which were developed from *Rpv3.1* resistant gene region, were used for PCR amplifications (Table 1). PCR was performed in a reaction volume of 10 µl containing 15 ng of DNA, 5 pmol of each forward and reverse primer, 0.5 mM of dNTP, 0.5 unit of GoTaq DNA Polymerase (Promega, Madison, WI) that included 1.5 mM of $MgCl_2$. PCR amplifications were performed on a Biometra Uno Thermocycler (Biometra, Göttingen, Germany) programmed as follows: initial denaturation step at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 50-60 °C (depending on each primer pair-specific annealing temperature) for 1 min and at 72 °C for 2 min with a final extension at 72 °C for 10 min.

Determination of fragment sizes

Fragment sizes of PCR products were determined by Fragment Analyser (Advanced Analytical Technologies, Inc. IS, USA) and allele size of the obtained peak was determined by ProSize software (Data Analysis Software ver. 3.0.1.6).

Artificial inoculation procedure and determination of resistance of individuals

Artificial inoculation and disease evaluation were carried out as defined by Özer *et al.* (2021). An isolate of the pathogen was obtained from a single sporulating (oil spot) lesion on a leaf of cv. 'Cabernet Sauvignon', which is highly susceptible to downy mildew. Resistance level of each individual was scored according to Boso *et al.* (2014).

False positive/negative rate

The resistant phenotype without the relevant allelic variant was considered false negative, and the susceptible phenotype with the relevant allelic variant was considered false positive. False negative and false

positive detection rates were calculated as the ratio of the number of false positive or false negative samples to the total number of samples from the population.

Results and Discussion

In the present study, a total of 1050 hybrid plants were acquired after cross 'Alphonse Lavallée' × 'Regent' cultivars in the breeding studies to develop new grapevine cultivars resistant to mildew. Some of these plants dried during germination or transition to field and did not develop; therefore, artificial mildew inoculation tests were carried out on 869 hybrid plants which matured vigorously.

Resistance scores of the hybrid plants after inoculation tests were determined as defined by Boso *et al.*, (2014). The resistance scoring defined 61 individuals as extremely resistant (7.01%), 43 as highly resistant (4.9%), 101 as resistant (11.62%), 186 as susceptible (21.40%), 96 as highly susceptible (11.04%), and 382 individuals as extremely susceptible (44.18%) (Özer *et al.*, 2021). Sporulation area after artificial inoculation in 'Alphonse Lavalleé', 'Cabernet Sauvignon' and hybrid genotypes are given in Figure 1. Susceptible cultivars exhibited dense and large sporulation area (Fig 1 A and B) in contrast to resistance genotypes with different levels (example genotypes number 340 and 534). These genotypes were extremely resistant (ER) since the pathogen could not grow on their leaves (Fig 1 C and D). The allele size of these genotypes was GF 18-08/410 bp which is evaluated as a more associated marker for downy mildew resistance (detailed data are given below).

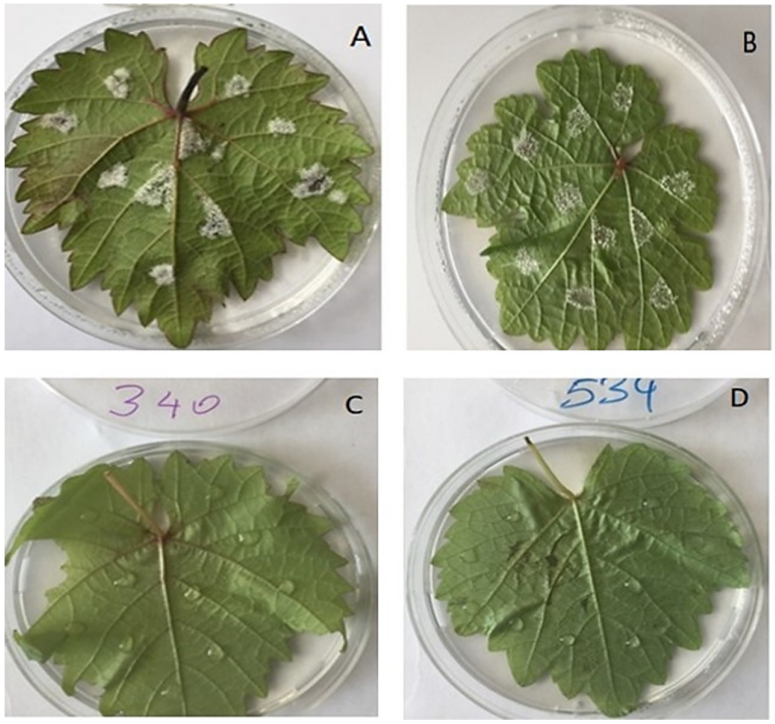

Figure 1. Sporulation area after inoculation of the pathogen in 'Alphonse Lavalleé' (A), 'Cabernet Sauvignon' (B), and hybrid genotypes (C and D)

DNA samples of the hybrid plants were amplified in PCR condition using GF 18-06 and GF 18-08 primers, and allele sizes were determined by ProSize software. The results were compared to confirm the relationship between the resistance scores of the offspring and the allele sizes of the genotypes.

The allele sizes of the genotypes, which were selected as resistant after the pathogen inoculation and the resistance levels of the genotypes (ER: extremely resistant; HR: highly resistant; R: resistant, respectively) are given in Table 1.

Using GF 18-06 marker, allele sizes were obtained as "385-390-407 bp" in the resistant parent 'Regent' carrying *Rpv 3.1* gene region and as "396-417 bp" in the susceptible 'Alphonse Lavalleé'. It may be seen that 57 genotypes which have 385 bp allele among 'Regent' alleles are defined to be susceptible to mildew at varying levels (8.86% false positive) when resistance levels are evaluated. 390-bp 'Regent' allele were defined in 58 susceptible genotypes (9.02% false positive) and 407-bp 'Regent' allele in 244 susceptible genotypes (37.94% false positive). No false negative results were detected (Table 1).

Zyprian *et al.* (2016) reported only paternal 387-bp allele associated to downy mildew resistance in QTL map acquired from 'GF.GA-47-42' (maternal genotype) x 'Villard blanc' (paternal genotype; Seibel 6468 × Seibel 6905; syn. 'Subereux') cross combination with GF 18-06 marker. Although 387 bp alleles were not detected in our research, it was evaluated that 390 bp and 385 bp 'Regent' alleles with low false-positive rates could be used in crossing populations for downy mildew resistant breeding.

The results show that the resistant parent 'Regent' gave allele sizes between "399-410 bp" and the susceptible parent 'Alphonse Lavallee' between "406-417 bp" by using GF 18-08. Only 32 genotypes, which had 410 bp alleles with GF 18-08 marker, were scored as susceptible on the resistance scale (4.97% false positive) (Table 1). This result was interpreted as that GF 18-08 marker has a strong relationship with downy mildew resistance and would be more appropriate to be used for MAS. A total of 307 individuals with 399 bp alleles were determined as susceptible (47.74% false positive) after scoring and were not associated with downy mildew resistance.

Percentages of resistant genotypes determined by resistance-associated alleles are given in Figure 2. Out of a total of 145 genotypes that gave amplification product with the GF 18-08 marker, 113 were determined resistant as a result of resistance level. 17 of 74 genotypes (22.97%) with GF 18-06/385 bp allele and 68 of 126 genotypes (53.96%) with GF 18-06/390 bp allele were found to be resistant after inoculation. The results show that GF 18-08/410 bp 'Regent' allele is the strongly associated marker for downy mildew resistance with a 77.93% percentage. GF 18-06/390 bp allele was found to be the second associated marker with a 53.96% percentage. Other 'Regent' alleles were not evaluated to be associated with downy mildew resistance (Figure 2).

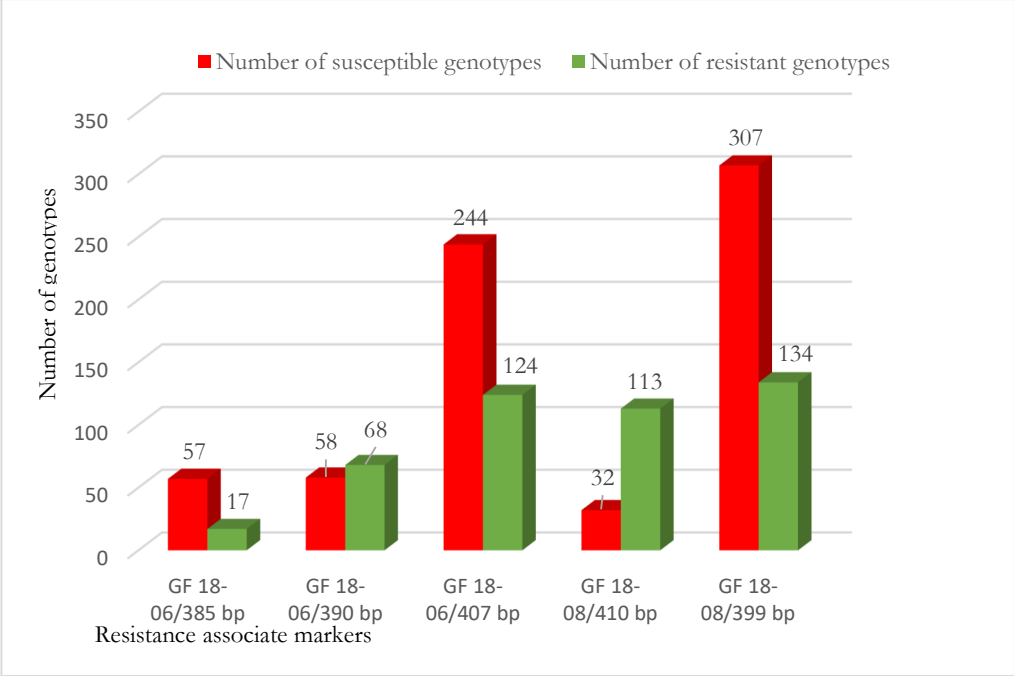

Figure 2. Percentages of resistant genotypes determined by resistance-associated alleles

GF 18-06 and GF 18-08 SSR primers were developed from 12X reference map of the grapevine in associated with *Rpv3.1* gene region (Schwander *et al.*, 2012; Zyprian *et al.*, 2016). *Rpv3.1* locus in 'Regent' map (Welter *et al.*, 2007) was verified in 'Regent' × 'Red Globe' genetic map developed by Van Heerden *et al.*, (2014) (Zyprian *et al.*, 2016). VMC7F2 SSR marker was mapped in the same region with GF18-08 on the chromosome 18 in the genetic map developed by Van Heerden *et al.* (2014). GF18-08 marker was specifically redesigned for the sequence that encircles the same SSR region as VMC7F2 (Zyprian *et al.*, 2016).

Table 1. Allele sizes for hybrid genotypes selected as resistant after artificial inoculation of *P. viticola* in 'Alphonse Lavaleé' × 'Regent' population and their resistance levels

Genotip No.	Allele size		Resistance level
	GF 18-06	GF 18-08	
Regent	385-390-407	399-410	HR
A. Lavalleé	396-417	406-417	ES
1	407-417	406-410	R
2	407-417	399-406	R
4	390-407	399-410	ER
7	390-396	399-410	ER
8	396-407	399-410	R
14	396-407	410-410	R
18	390-407	406-410	R
37	407-417	399-410	R
39	407-417	406-410	R
52	396-407	399-406	R
56	390-	410-	R
60	396-407	399-410	R
64	390-396	406-410	R
68	390-396	399-410	ER
80	*-417*	*399-406*	R
81	390-396	406-410	R
89	390-407	399-410	R
98	396-407	399-410	R
100	407-417	399-406	R
104	407-417	399-406	HR
105	390-396	410-	HR
120	390-	406-410	ER
126	390-396	399-406	R
132	390-396	410-	R
140	407-417	399-	R
148	396-407	399-410	R
149	407-417	406-410	HR
158	407-417	399-417	ER
161	407-417	399-406	R
164	385-390	399-406	ER
168	396-407	399-410	HR
170	390-407	399-410	HR

171	396-417	399-410	ER
175	407-417	410-	HR
178	390-	399-410	ER
180	390-396	399-410	ER
183	390-396	406-410	R
187	390-	399-410	ER
188	396-407	399-410	R
189	396-407	399-410	ER
199	396-407	406-410	ER
205	390-396	-	HR
210	396-407	399-406	ER
211	390-407	399-410	ER
215	390-407	399-410	ER
217	390-407	399-410	ER
219	390-396	399-410	ER
223	396-407	406-410	R
225	407-417	399-410	R
229	396-407	406-410	R
234	390-407	399-410	ER
236	390-	399-410	R
238	390-396	399-410	HR
241	407-417	399-410	R
245	396-407	-	HR
249	396-407	399-410	HR
254	396-407	399-406	R
258	390-407	399-410	R
261	390-396	406-410	HR
263	390-396	399-410	R
264	390-396	406-410	R
266	390-396	410-	R
267	396-407	406-410	R
269	407-417	399-410	R
271	407-417	399-417	R
277	407-417	399-410	R
281	396-407	399-410	ER
283	396-407	399-410	R
288	396-407	399-406	R
289	396-407	399-406	R
290	390-396	399-410	R
291	390-407	410-410	R
301	396-407	399-406	R
307	390-396	406-410	R
309	385-390	406-410	R
315	385-390	410-417	ER
317	396-407	399-406	R

319	390-396	406-410	HR
321	385-390	406-410	HR
322	390-407	399-417	HR
323	385-390	406-?	R
338	407-417	399-406	ER
340	407-413	399-406	ER
341	-407	399-406	R
342	-407	406-?	HR
344	407-417	399-410	ER
345	407-413	399-406	HR
349	390-407	399-406	HR
352	396-407	399-406	R
353	390-396	399-410	R
355	390-396	399-410	R
359	396-407	406-410	R
370	407-417	399-406	R
372	390-390	399-410	R
379	407-417	399-410	R
387	*396-417*	399-406	R
388	385-396	399-410	R
390	396-407	399-410	R
392	407-417	399-410	R
393	390-396	399-410	R
397	-417	410-	R
399	396-407	399-417	R
401	390-396	399-417	R
409	385-390	399-410	ER
410	396-407	406-410	R
412	385-396	399-417	HR
417	407-417	399-406	R
420	407-417	399-399	R
423	-407	399-410	ER
428	407-417	-	HR
430	396-407	410-	HR
431	390-396	399-410	R
434	407-417	399-399	R
435	396-407	399-410	R
436	407-417	399-410	R
437	396-407	399-410	R
439	407-417	410-417	R
441	407-417	410-	R
442	407-417	399-410	R
443	385-396	410-	HR
444	396-407	406-?	R

446	385-390	399-	R
449	407-417	399-410	R
452	385-390	399-406	R
457	385-396	410-	R
459	385-390	406-?	HR
461	407-417	399-406	ER
463	396-407	399-410	R
469	407-417	399-417	R
470	407-413	399-406	ER
472	385-390	399-410	R
473	390-407	410-	R
474	390-396	399-410	HR
475	*396-417*	399-410	R
480	396-413	399-406	R
481	390-396	399-406	R
483	396-407	399-410	R
485	407-417	406-?	ER
492	407-417	399-410	R
495	385-390	399-406	HR
497	390-396	399-406	R
500	407-417	399-410	R
505	396-407	399-406	ER
507	396-407	399-410	R
515	396-407	410-	ER
519	396-407	406-410	R
522	396-407	399-	HR
524	407-417	399-406	R
532	396-407	406-410	R
534	384-390	410-	ER
535	390-407	399-417	ER
538	396-407	399-406	R
539	396-407	399-406	R
542	396-407	-	ER
543	396-407	399-410	R
547	390-396	399-410	R
549	385-390	399-410	ER
553	390-407	410-	R
558	407-417	399-410	R
560	390-407	399-410	R
568	396-407	-	R
570	396-407	399-417	R
571	396-407	399-406	R
572	396-407	410-417	ER
573	-417	410-	R
574	407-417	410-417	ER

575	385-396	410-	ER
580	390-396	399-410	ER
582	407-417	399-	ER
586	396-407	399-410	ER
587	396-407	410-	ER
588	390-407	399-410	ER
590	390-396	399-410	R
592	390-	399-410	ER
594	396-407	-	HR
599	407-417	399-410	R
600	396-407	399-417	R
601	390-	399-410	R
602	396-407	399-410	R
604	396-407	399-399	R
612	396-407	399-406	R
613	385-390	399-406	R
624	407-417	399-417	R
649	396-407	399-410	R
651	*396-417*	399-410	R
661	407-417	410-	R
673	407-417	399-417	R
823	407-417	-	R
825	407-417	399-406	R
828	407-417	399-399	R
915	407-417	399-406	R
921	396-407	399-	R
932	407-417	-	R
942	396-417	399-410	R
971	396-407	399-406	R

(ER: extremely resistant; HR: highly resistant; R: resistant).

Zyprian *et al.* (2016) found the allele with "399 bp" size as associated with resistance using the GF 18-08 marker. "393-399 bp" allele distribution was acquired in 'Regent' with GF 18-08 primer. The alleles for downy mildew resistant/tolerant hybrids are 'Villard Blanc' (383-399 bp), Seibel 6468 (387-399 bp), Suberux (383-399 bp), and GF.GA 47-42 (389-392 bp). In the present study, 'Regent' gave amplification product with "399-410 bp" size. However, 399 bp 'Regent' allele, in contrast to the findings above, was not associated with resistance while 410 bp allele was found to be highly related to downy mildew resistance. It is thought that this difference may be due to the possible differences encountered during the determination of fragment sizes with fragment analyzers. Therefore, it is highly likely that 399 bp allele associated with resistance in the study by Zyprian *et al.*, (2016) corresponds to 410 bp allele in the present study.

The application of MAS in resistance breeding studies provides researchers with useful perspectives from many different aspects. Genotyping genetic resources with molecular markers related to important traits enables the identification of optimized crossover combinations. For instance, MAS may allow target parent selection with the potential to combine different resistance loci in offspring to increase resistance level and sustainability of resistance with regard to resistance to downy mildew (Eibach and Töpfer, 2015). However,

phenotyping remains essential to characterize host-pathogen interaction and to evaluate the effective resistance level of new varieties (Possamai *et al.*, 2020).

On the other hand, it is crucial for early selection with MAS to be effective to select molecular markers that are closely associated with the characteristics examined in the selected parents in hybridization combinations. Kuchel *at al.* (2007) stated that the frequency of Lr34/Yr18 rust-resistance genes increased from 0.25 to 0.60 with MAS in BC1 wheat population, reported, however, the increase as from 0.25 to only 0.27, and claimed that it resulted from the actually poor relationship between Lr34/Yr18 genes and the markers used in the scanning of BC1 plants (Bernardo, 2008). Similarly, Yıldırım *et al.* (2019) determined that some grapevine genotypes collected from the Black Sea coast with high humidity and precipitation, which they determined to have resistance-associated alleles as a result of MAS, showed intense mildew symptoms after artificial inoculation. Researchers point out the importance of validating the results of the marker used for MAS with the results of artificial/natural inoculation. Therefore, it is extremely important to validate the selected molecular markers for MAS in different genotypic sources.

Conclusions

The development of new downy mildew resistant cultivars through conventional breeding provides an effective solution for disease management. In such studies, MAS provides rapid and cost-effective genotyping methods. In the present study, it was determined that 385 bp and 390 bp alleles which were acquired with GF18-06 marker associated with *Rpv3.1* gene region resulted in 8.86% and 9.02% false positives, respectively, and that 410 bp allele among 'Regent' alleles acquired with GF 18-08 marker had high correlation with downy mildew resistance based on resistance scoring results. Therefore, GF 18-08 marker is recommended for MAS in downy mildew resistant grape breeding studies.

Authors' Contributions

Conceptualization: MA and İŞ; Data curation: MA and BA; Formal analysis: İS and NÖ; Methodology: MA, İŞ, BA, NÖ; Supervision: HİU; Validation: İS and NÖ; Writing – original draft: MA and İŞ; Writing – review & editing: NÖ and HİU.

All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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