Vitis **61**, 71–76 (2022) DOI: 10.5073/vitis.2022.61.71-76

The effect of homozygosity of locus *Rpv12* on downy mildew resistance of grapevine leaves

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Summary

Inoculation trials with different pathogenic fungi and oomycetes have not yet shown any significant effect of homozygosity in resistance loci on the level of resistance. However, here a considerable reduction in mycelial growth of the oomycete *P. viticola* was found in association with the homozygous state of the *Rpv12* locus. This effect was detected by aniline blue staining of the mycelium after experimental inoculation of leaf tissue and quantified by image analysis. Genotypes homozygous for the *Rpv12*-locus as classified by SSR marker data inhibited mycelial growth considerably more in comparison to genotypes carrying the locus *Rpv12* in heterozygous state.

Key words: aniline blue staining; combined resistance loci; LSH (locus specific homozygosity); mycelial development, resistance to *Plasmopara viticola* (*Rpv*), SSR Marker; homozygosity.

Introduction

The pathogen *Plasmopara viticola,* the oomycete causing downy mildew of grapevine, was introduced into European viticulture during the 19th century (Viala 1893). Since that time, attempts to strengthen the plants defense mechanisms by grapevine breeding aim to introgress resistance traits into noble European *Vitis vinifera* quality cultivars (Töpfer *et al.* 2011a). For this purpose, accessions of American and Asian wild grapevine species such as *V. rupestris*, *V. riparia*, *V. cinerea, V. piasezkii, V. amurensis* and *Muscadinia rotundifolia* are used as resistance donors (Wan *et al.* 2007, Maul *et al.* 2021). To date, 27 loci of resistance to *Plasmopara viticola* (*Rpv*) have been described and genetically mapped, largely originating from germplasm of these wild species (Maul *et al.* 2021). The loci *Rpv1*, *Rpv3* (*Rpv3.1*, *Rpv3.2*, *Rpv3.3*), *Rpv10* and *Rpv12* have been used predominantly for breeding purposes of grapevine cultivars resistant to downy mildew (Maul *et al.* 2021). It is generally assumed that genes encoding proteins with NLR motifs (nucleotide binding site and leucine rich repeats) play an important role in the resistance pathway, acting as receptors of pathogen strain-specific effectors that trigger the response of the host plant upon interaction (Lai and Eulgem 2018). Loci *Rpv1* and *Rpv3* have been studied in detail. For *Rpv1*, a TIR-NBS-LRR motif was identified in a resistance-associated defense protein gene (Qu *et al*. 2021). For *Rpv3*, an NLR motif was described as important element of the resistance-associated putative receptor gene (Foria *et al.* 2020, Qu *et al*. 2021). Grapevine breeding in the future might also be facilitated by crossing locus-specific homozygous (LSH) genotypes, since all progeny would inherit a copy of the resistance locus. However, little is known about the effects of homozygosity of various resistance loci on the level of pathogen resistance. To the best of our current knowledge, this has only been investigated in a study reported by Dry *et al.* (2017) using the *Ren4* locus, mediating resistance against *Erysiphe necator*. In this case, the homozygous *Ren4* carriers showed a slightly faster induction of programmed cell death as a defense reaction to the obligate biotrophic ascomycete *E. necator* as compared to the heterozygous situation. In this present work, the impact of homozygosity of the *Rpv12* locus on the spread of mycelial growth in grapevine leaves was examined by aniline blue staining and microscopy.

Material and Methods

Plant material and DNA extractions: Leaf material from the following genotypes was used to evaluate resistance: Two genotypes carrying *Rpv12* in the homozygous state, Hozy01 and Hozy10, provided by the breeding department of the institute (R. Eibach and O. Trapp), formed the core of this work. Hozy01 carries the *Rpv12* locus homozygously and no other known resistance locus, while Hozy10 carries *Rpv12* and *Rpv3.1* both in the homozygous situation. Both genotypes emerged from the parental genotype IRZ0973 by self-pollination and were characterized using SSR-marker genotyping (see below). The sampled plant material was taken from the Grapevine Germplasm Repository at the Institute for Grapevine Breeding Geilweilerhof (49°12'54.1"N, 8°02'41.3"E). It was propagated through dormant 2-bud cuttings in the greenhouse. Furthermore, progeny from open pollination of 'Kunbarat' (29 genotypes) were analysed with regard to the effect of *Rpv12* homozygosity. 'Kunbarat' open pollinated progeny were taken from seeds produced on greenhouse plants. Marker data showed that these genotypes are a self-progeny.

DNA extraction and SSR-marker analysis: The extraction of DNA to check the presence of resistance loci by locus-linked SSR (simple sequence re-

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peat) markers was done using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

For SSR marker analysis, the markers GF18-06 & UDV-737 (*Rpv3.1* & *Rpv3.2*), GF18-01 & GF18-04 (*Rpv3.3*), GF09-48, GF09-46 (*Rpv10*) as well as sc81_7.4, UDV-350, UDV-343, GF14-24, UDV-340, UDV-360, UDV-370, and VMC2H12 (*Rpv12*) were used in multiplex PCR assays with Kapa 2G polymerase (Kapa Biosystems, Inc., Hoffmann-La Roche). The sequences of the primer pair flanking SSR marker VMC2H12 are available in the repository of the National Centre for Biotechnology Information (NCBI GenBank/Nucleotide). Primer pairs Sc81_7.4, UDV-350, UDV-343, UDV-340, UDV-360 and UDV-370 were published by Venuti *et al*. (2013) and UDV-737 was described by Di Gaspero *et al*. (2012). Markers GF18-01, GF18-06, GF09-48, GF09-46 were published by Schwander *et al.* (2012) and GF14-24 was described by Zyprian *et al.* (2016).

Primers were purchased from Metabion International, Planegg, Germany. Allele length analysis of their amplification products was carried out according to Müllner *et al.* (2020).

Alkaline aniline blue staining: Intercellular mycelial growth was followed by alkaline aniline blue staining according to Hood and Shew (1996). Leaf discs were incubated for at least 3 h in 1 N KOH solution at 65 °C. Afterwards, the leaf discs were washed in H_2O_{deion}, transferred to a microscope slide, stained with 50 µL of an aniline blue solution (0.05 % aniline blue in 0.067 M K_2HPO_4) and a coverslip was placed on top. After 10 min, the dye was replaced with water by carefully pipetting H_2O_{deion} until no more streaks were visible. The samples were documented by fluorescence microscopy (DM4000B, Leica, Wetzlar) using a GFP filter (Leica A, Excitation λ=340-380 nm, dichroitic mirror 400 nm, Emission: LP 425).

Comparison of mycelial growth using Fiji and R: To compare the mycelial growth of homozygous and heterozygous *Rpv12* carriers, three leaf discs each of three leaves of every genotype (in total nine leaf discs per genotype) were investigated and compared to the heterozygous *Rpv12* carrier 'Kunbarat' (*V*IVC No. 6557) and the susceptible grapevine cultivars 'Italia' (*V*IVC No. 5582) and `Afus Ali´ (*V*IVC No. 122). Experimental inoculation used zoospores released from a 20.000 sporangia·mL^{-1} solution. To allow discharge of zoospores, sporangia were kept in H_2O_{deion} for approximately 90 min and afterwards filtered to remove empty sporangia using aquarium filter cotton, which previously was washed once with isopropanol, twice with H_2O_{deion} and dried overnight (Günther Buchholz, pers. communication). The leaf discs with a diameter of 1.3 cm were inoculated by pipetting 40 µl zoospore-solution on the abaxial side. The leaf discs were placed on a square plate (243 mm x 245 mm, Corning Incorporated, Corning, NY, USA) filled with 1 % Agar (Gustav Essig GmbH & Co. KG, Mannheim). After 72 hpi (22 °C, 16 h light, 8 h darkness) every leaf disc was stained as described above. Five pictures of every leaf disc were taken using 100 x magnification and the GFP-Filter (in total 45 images per genotype). Because there was so little mycelial growth on Hozy01 and Hozy10, the leaf veins were manually removed from the images using paint.net (Version 4.2.14) to facilitate the evaluation. The images were further analysed using Fiji (ImageJ-win64, Schindelin *et al.* 2012). Different conditions in Fiji had to be used for the different genotypes as specified in the Macro Code (attached in supplements) due to the intensity of mycelia. The macros created binary images, which represent mycelium in white and background in black. The program counted the number of white pixels and calculated the percentage of the total pixel amount. Results were transferred to an Excel-Sheet and subjected to statistical analysis using R (Version 4.0.3; R Core Team, 2020; https://www.R-project.org/). The normal distribution was analysed using Shapiro's test and, if necessary, transformed using log for left shift and sqrt for right shift to ensure comparability. The transformation of each genotype was compared to the data from the grapevine cultivar 'Italia' which was used as susceptible reference genotype. The significance was then determined using Welch's t-test and for comparison of Hozy01 to Hozy10 supported by Mann-Whitney U-test.

Results and Discussion

Little is known about possible resistance enhancement through homozygosity of resistance loci. To date, locus-specific homozygous (LSH) genotypes might be used in grapevine breeding to ensure the inheritance of resistance loci without the necessity to check the progeny of controlled crosses with resistance-linked molecular markers. In general, the breeding of largely homozygous genotypes is avoided due to high inbreeding depression commonly observed in grapevine (Alleweldt and Possingham 1988, Töpfer *et al.* 2011b).

To ensure that the genotypes investigated do not carry any other known *P. viticola* resistance locus, comparative SSR marker analyses were carried out. The references included the genotypes IRZ0973 (parental genotype of Hozy01 and Hozy10, carrying *Rpv3.1* heterozygously and *Rpv12* partially homozygously), 'Merzling' (*V*IVC No. 4251, *Rpv3.3*, *Ren3*, *Ren9,* Resistance to *Erysiphe necator*), 'Regent' (*V*IVC No. 4572, *Rpv3.1*, *Ren3*, *Ren9*) and 'Solaris' (*V*IVC No. 20340, *Rpv3.3*, *Rpv10*, *Ren3*, *Ren9*) (data not shown). These data provide evidence that Hozy01 carries the *Rpv12* locus homozygously, whereas Hozy10 carries both *Rpv12* and *Rpv3.1* in homozygous state (Tab. 1).

Leaf disc inoculation tests followed by microscopic analyses showed that the genotypes Hozy01 and Hozy10 carrying *Rpv12* in the homozygous state exhibited a strong reduction of *P. viticola* mycelial growth. Staining with alkaline aniline blue revealed a drastic reduction of mycelial development and almost no mycelium was detectable (Fig. 1). This was quite in contrast to heterozygously *Rpv12*-carrying genotypes such as 'Kunbarat', which showed 48.4 %, reduction of mycelial growth at 72 hpi as compared to the susceptible controls 'Afus Ali' and 'Italia' (see Fig. 2).

Two susceptible genotypes served as references: 'Afus Ali', which was previously identified as a parental genotype of 'Kunbarat', and 'Italia', mistakenly considered as a parental genotype of 'Kunbarat' (Müllner *et al.* 2020). 'Italia' is highly susceptible and grows well in greenhouse

Table 1

Detection of the *Rpv3* and *Rpv12* loci in the LSH genotypes Hozy01 and Hozy10. SSR marker designations, the genotypes Hozy01, Hozy10 and IRZ0973 and associated SSR marker data, the associated locus description and the resistance associated allele sizes are indicated. The marker position based on the reference genome PN40024 12x.v2 (JAILLON *et al*. 2007, CANAGUIER *et al*. 2017) is provided. The *Rpv3* locus is differentiated into the variants *Rpv3.1*, *Rpv3.2* and *Rpv3.3*. Some markers are listed twice as they indicate different loci depending on their allele size. Numbers in bold indicate correspondence to the resistance-associated allele sizes

Marker	Hozy01	Hozy10	IRZ0973	Position PN40024 12x.v2	Locus	Allele size [bp]
GF18-06	386	**388**	386/**388**	chr18_29340182	*Rpv3.1*	388
UDV-737	296	**282**	**282/296**	chr18_29467530	*Rpv3.1*	282
GF18-06	386	388	386/388	chr18_29340182	*Rpv3.2*	407
UDV-737	296	282	282/296	chr18_29467530	*Rpv3.2*	302
GF18-01	280	276	276/280	chr18_28615897	*Rpv3.3*	278
UDV-737	296	282	282/296	chr18_29467530	*Rpv3.3*	274
GF09-48	336	336/348	336/348	chr09_3854406	*Rpv10*	360
GF09-46	408	408/418	408/418	chr09_3666103	*Rpv10*	416
Sc81_7.4	**276**	**276**	**276**/334	chr14_8426890	*Rpv12*	276
UDV-350	**310**	**310**	303/**310**	chr14_8963923	*Rpv12*	310
UDV-343	**160**	**160**	**160**	chr14_9012000	*Rpv12*	160
GF14-24	**261**	**261**	**261**	chr14_9026791	*Rpv12*	261
UDV-340	**178**	**178**	**178**	chr14_9145665	*Rpv12*	178
UDV-360	**208**	**208**	202/**208**	chr14_9910488	*Rpv12*	208
UDV-370	**198**	**198**	192/**198**	chr14_10247617	*Rpv12*	198
VMC2H12	**100**	**100**	**100**/111	chr14_10304031	*Rpv12*	100

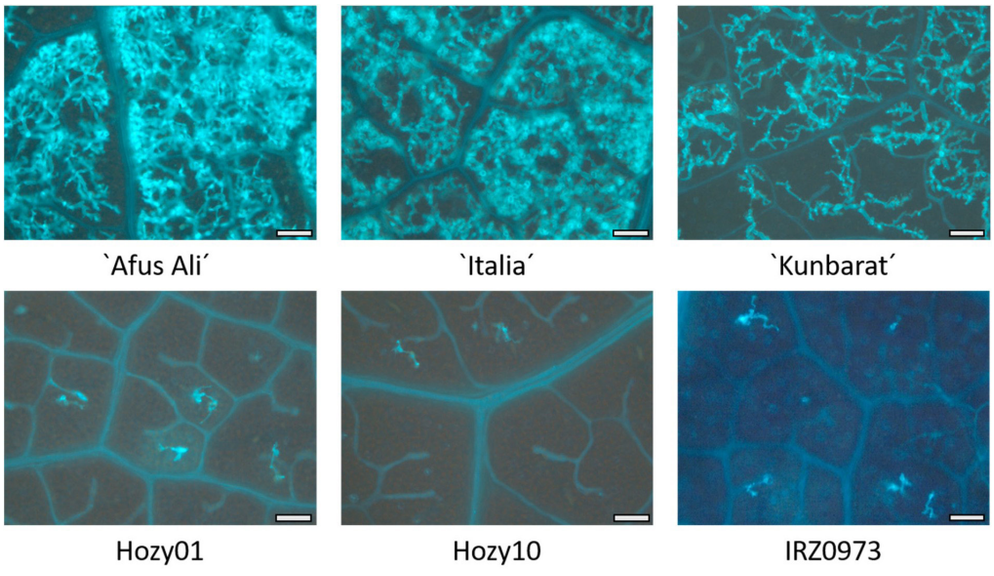

Fig. 1: Alkaline aniline blue staining of *P. viticola* mycelial growth in leaves of different genotypes at 72 hpi. 'Afus Ali' and 'Italia' served as susceptible reference genotypes. 'Kunbarat' carries *Rpv12* heterozygously, Hozy01 carries *Rpv12* homozygously and Hozy10 carries both *Rpv3.1* and *Rpv12* homozygously. The parental genotype of Hozy01 and Hozy10, IRZ0973, carries *Rpv3.1* heterozygously but *Rpv12* partially homozygously. The image section is 871.00 x 653.25 µm. The scale bar corresponds to 100 µm.

conditions. The genotype 'Afus Ali' first had to be evaluated as "non-normally distributed" as the data of one of the leaves diverged largely, possibly due to the presence of spray residues on the leaves. 'Afus Ali' is a *V. vinifera* grapevine cultivar and accordingly susceptible to *P. viticola*. For this genotype, a normal distribution was established by omitting data of this one deviating leaf. The corresponding boxplot of mycelial development with the corrected 'Afus Ali' data set as well as 'Italia', 'Kunbarat', Hozy01 and Hozy10 is shown in Fig. 2. The significance of variation in

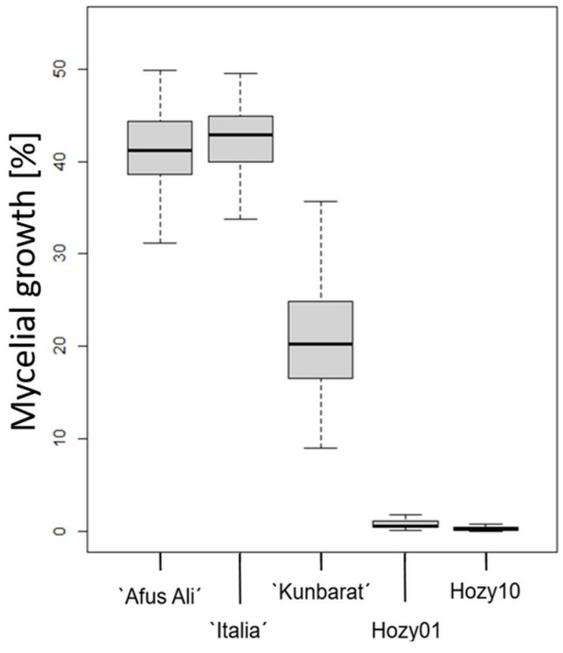

Fig. 2: Comparison of mycelial growth of various *Rpv12*- carrying and non-carrying genotypes. The graphical representation of the mycelial growth calculation using Fiji and R is presented. In total, 45 images of each genotype were taken after 72 hpi for analysis. 'Afus Ali' (41.2 %) and 'Italia' (42.7 %) were provided as susceptible control genotypes, 'Kunbarat' (20.8 %) carries the *Rpv12* locus, Hozy01 (0.7 %) carries *Rpv12* homozygously and Hozy10 (0.39 %) carries *Rpv12* as well as *Rpv3.1* homozygously. The mycelial growth of all genotypes differs significantly from 'Italia' except 'Afus Ali'.

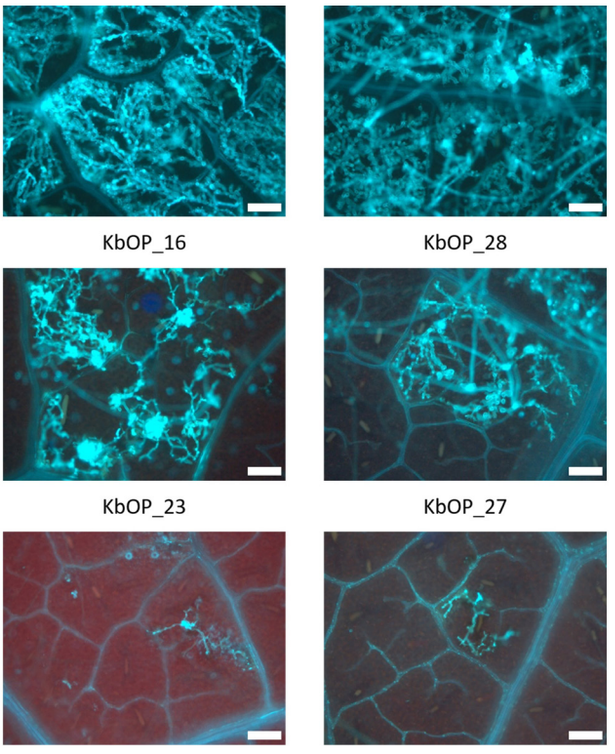

Fig. 3: Alkaline aniline blue staining of 'Kunbarat' progenies infected with *P. viticola* at 8 dpi. The genotypes KbOP_16 and KbOP_28 don't carry *Rpv12*, the genotypes KbOP_23 and KbOP_27 carry *Rpv12* heterozygously and the genotypes KbOP_07 and KbOP_29 carry *Rpv12* homozygously. The scale bar corresponds to 100 µm.

mycelial development was determined with regard to the susceptible grapevine cultivar 'Italia' by means of Welch's t-test. It was demonstrated that, with respect to 'Italia', all *Rpv12* carrying genotypes showed a significant reduction in mycelial growth.

The mycelial growth of *P. viticola* in Hozy01 accounts for only about 1 % of the microscopic image area, while it is even more and significantly reduced in Hozy10 to 0.5 %. However, it should be noted that the parental genotype IRZ0973 also shows a strongly reduced mycelial development (Fig. 3), as it carries the loci *Rpv3.1* (heterozygous) and *Rpv12* (partially homozygous). A combination of these loci has already been recognized to raise an additive effect (Venuti *et al.* 2013, Eisenmann 2019 Diss.). Meanwhile it became evident that the genotypic background may have an influence on the expression level of resistance, even if no further loci are detectable (Foria *et al.* 2017).

To verify the effect of homozygosity of *Rpv12* on *P. viticola* mycelial growth, a progeny resulting from open pollination of 'Kunbarat' was tested. This open pollination progeny (OP, which actually is a self-progeny as indicated by SSR marker data, Tab. 2) consisted of 29 genotypes. Five of them carried no *Rpv12*-locus, ten were homozygous in the *Rpv12*-locus, as estimated by markers UDV-340, UDV-343, UDV-350, UDV-360, UDV-370 and VMC2H12. One additional genotype was homozygous in marker UDV-370 and another one in marker UDV-340. Eleven genotypes were heterozygous in all tested markers (Tab. 2). The phenotypes of these genotypes were tested twice by leaf disc inoculation assay and once (three leaf discs) by additional fluorescence microscopy at 8 dpi. Fourteen genotypes showed a strong mycelial growth, while ten showed a reduced growth. Out of the eleven *Rpv12*-homozygous genotypes, seven showed a strong inhibition of mycelial growth. Two additional homozygous genotypes showed nearly no formation of sporangiophores (Fig. 3).

One may consider the possibility that the structure of the leaf or a difference in nutrient supply of the plants affects the susceptibility of the genotype. After all, the 'Kunbarat' OP progeny plants are inbred genotypes and inbreeding depression is very common in grapevine (Alleweldt and Possingham 1988, Töpfer *et al.* 2011b). However, after planting in rhododendron soil (Hawita Gruppe GmbH, Vechta, Germany; elevated nutritional value and acidic pH), the plants all proved to be vigorous and deeply green, with the exception of three variegated genotypes (KbOP_03 and KbOP_09 which are homozygous in the *Rpv12*-locus and KbOP_05 which is heterozygous in the *Rpv12*-locus).

The reduced mycelial growth of *P. viticola* can be attributed to a dosage effect. There are 12 NBS-LRR (nucleotide binding side leucine rich repeat) structures encoded at the *Rpv12* corresponding locus in the reference genome of PN40024 12x.v2 (Venuti *et al.* 2013, Müllner 2021). It is known that NBS-LRR structures play an essential role in defense against pathogens such as *P. viticola*. Kortekamp *et al.* (2008) showed that after a *P. viticola* infection of the resistant cultivar *V. riparia* 'Gloire de Montpellier' (*Rpv5*, *Rpv6*) a specific expression of *VRP1*, a *CC-NBS-LRR*-Gene

Table 2

SSR marker analysis of the 'Kunbarat′ open pollination lines. The names of the genotypes are listed (where KbOP stands for 'Kunbarat' open pollination), the marker designations, the position in the genome (based on the reference genome PN40024 12x.v2, Jaillon *et al*. 2007, Canaguier *et al*. 2017) and the resistance-associated SSR allele sizes [bp] are provided. Besides OIV (inverse) is listed: 1 for no Sporangiphores and 9 for highly susceptible. Genotypes carrying the *Rpv12* locus are written in bold

	UDV-350	**UDV-343**	**UDV-340**	**UDV-360**	**UDV-370**	**VMC2H12**	**OIV (inverse)**
	Chr.14: 8963923	**Chr.14: 9012000**	**Chr.14: 9145665**	**Chr.14: 9910488**	**Chr.14: 10110182**	**Chr.14: 10304031**	
Rpv12	**310**	**159**	**179**	**208**	**198**	**100**	
KbOP_01	308 / 310	159 / 191	177 / 179	204 / 208	196 / 198	100 / 106	5
KbOP_03	310 / 310	159 / 159	179 / 179	208 / 208	196 / 198	100 / 100	2
KbOP_04	308 / 308	191 / 191	177 / 177	204 / 204	196 / 196	106 / 106	contamination
KbOP_05	308 / 310	159 / 191	177 / 179	204 / 208	196 / 198	100 / 106	3
KbOP_07	310 / 310	159 / 159	179 / 179	208 / 208	198 / 198	100 / 100	1
KbOP_09	310 / 310	159 / 159	179 / 179	208 / 208	198 / 198	100 / 100	2
KbOP_10	308 / 310	159 / 191	177 / 179	204 / 208	196 / 198	106 / 106	1
KbOP_13	310 / 310	159 / 159	179 / 179	208 / 208	198 / 198	100 / 100	3
KbOP_14	308 / 310	159 / 191	177 / 179	204 / 208	196 / 198	100 / 106	3
KbOP_15	308 / 310	159 / 191	177 / 179	204 / 208	196 / 198	100 / 106	2
KbOP_16	308 / 308	191 / 191	177 / 177	204 / 204	196 / 196	106 / 106	7
KbOP_17	308 / 308	191 / 191	177 / 177	204 / 204	196 / 196	106 / 106	9
KbOP_18	310 / 310	159 / 159	179 / 179	208 / 208	198 / 198	100 / 100	2
KbOP_19	308 / 310	159 / 191	177 / 179	204 / 208	196 / 198	100 / 106	3
KbOP_20	308 / 310	159 / 191	177 / 179	204 / 208	198 / 198	100 / 106	2
KbOP_21	310 / 310	159 / 159	179 / 179	208 / 208	198 / 198	100 / 100	1
KbOP_22	308 / 308	191 / 191	177 / 177	204 / 204	196 / 196	106 / 106	8
KbOP_23	308 / 310	159 / 191	177 / 179	204 / 208	196 / 198	100 / 106	4
KbOP_24	308 / 310	159 / 191	179 / 179	204 / 208	196 / 198	100 / 106	4
KbOP_25	308 / 310	159 / 191	177 / 179	204 / 208	196 / 198	100 / 106	2
KbOP_26	310 / 310	159 / 159	179 / 179	208 / 208	198 / 198	100 / 100	1
KbOP_27	308 / 310	159 / 191	177 / 179	204 / 208	196 / 198	100 / 106	3
KbOP_28	308 / 308	191 / 191	177 / 177	204 / 204	196 / 196	106 / 106	contamination
KbOP_29	310 / 310	159 / 159	179 / 179	208 / 208	198 / 198	100 / 100	1
KbOP_30	310 / 310	159 / 159	179 / 179	208 / 208	198 / 198	100 / 100	1
KbOP_32	310 / 310	159 / 159	179 / 179	208 / 208	198 / 198	100 / 100	1
KbOP_34	308 / 310	159 / 191	177 / 179	204 / 208	196 / 198	100 / 106	5
KbOP_35	308 / 310	159 / 191	177 / 179	204 / 208	196 / 198	100 / 106	2
Kunbarat	308 / 310	159 / 191	177 / 179	204 / 208	196 / 198	100 / 106	5

is initiated. Additionally, Foria *et al.* (2020) demonstrated that the causative factor of the *Rpv3* transmitted hypersensitive response is mediated by two *TIR-NB-LRR*-genes (Chitarrhini *et al*. 2020). Besides, Chitarrhini *et al.* (2020) observed that ROS and salicylic acid production are elevated in an *Rpv12*-carrying genotype, a fact that they connect to a hypersensitive response initiated through *NBS-LRR* genes. Nevertheless, the data shown here in combination with microscopic observations indicate that not only the locus is relevant for resistance, but the genetic background also plays an important role. This has been pointed out by Foria *et al.* (2017) and may explain why most, but not every *Rpv12*-LSH genotype is highly *P. viticola* resistant.

Conclusion

This analysis indicates that *Rpv12*-locus-specific homozygosity may have a strong effect to reduce mycelial growth of downy mildew (*P. viticola*) and may be a promising possibility to enhance the genetic resistance of grapevine cultivars.

Acknowledgements

This research was supported by the European Fund for Regional Development (EFRE) in the frame of Interreg V "Vitifutur" and the Deutsche Forschungsgemeinschaft (German Research Foundation) DFG (Zy11/11-1). We wish to thank A. Abdaan for technical assistance, G. Buchholz for providing his method of zoospore separation from sporangia, L. Gebauer for statistical support, T. Proksch for helping with the macros for mycelial growth and the Julius Kuehn Institute, Institute for Plant Protection in Fruit Crops and Viticulture, for providing their fluorescence microscope.

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Received December 12, 2021
Accepted: June 2, 2022