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Verification of resistance loci pyramiding in popular interspecific grape varieties using SSR markers

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Abstract: Fungal pathogens *Plasmopara viticola* (Berk. & M.A. Curtis) Berl. & De Toni (downy mildew) and *Erysiphe necator* Schwein. (powdery mildew) represent the biggest threats for grape growers worldwide. Under suitable conditions, these pathogens can spread very quickly through vineyards and cause significant damage. The most ecological way to reduce the possibility of infection in vineyards is growing interspecific genotypes which are able to suppress these pathogens and stop the spread of infection. With this in mind, 9 international and 11 Czech grapevine genotypes were analysed in order to genotype the resistance loci present in them. As a tool a set of SSR markers linked to known loci of resistance to downy and powdery mildew was used. Namely, presence of four loci responsible for resistance to *Plasmopara viticola* (*Rpv3*, *Rpv4*, *Rpv7*, and *Rpv10*) and two for *Erysiphe necator* (*Ren3* and *Ren9*) were analysed with respective SSR markers. By this way the degree of resistance gene pyramiding was newly assessed in all analysed cultivars and their perspectives in grapevine breeding are discussed.

Keywords: downy mildew; *Erysiphe necator*; grapevine; *Plasmopara viticola*; powdery mildew; resistance

Downy and powdery mildew represent an annual threat in vineyards worldwide. These diseases are caused by pathogens *Plasmopara viticola* and *Erysiphe necator*, that are native to North America and were brought to Europe in the 19th century through propagation material. They infect all green parts of the vine. Under suitable conditions and strong infection pressure they can completely destroy leaves and then significantly decrease grape quality (Stummer et al. 2005). One of the most effective ways to protect vineyards is to use chemical plant protection products. These substances are often based on sulfur (effective against *E. necator*) and copper (effective against *P. viticola*) which may leave a residue in the soil for a long time (Mackie et al. 2012). In the case of copper-based chemical products, this is a significant environmental problem, based on the current Commission implementing regulation EU (2018, 1981) usage of copper in vineyard is limited by 28 kg copper per 7 years per hectare of vineyard. Fortunately, there is a more eco-friendly way to protect vineyards by using interspecific genotypes of grapevine. Here takes advantage of the fact that some American and Asian *Vitis* L. species have evolved resistant reaction

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to these two pathogens and can thus cope with the infection attack (Merdinoglu et al. 2018; Atak 2022) . The genomic regions which cause a resistance response are called *Rpv* (for downy mildew caused by *Plasmopara viticola*) and *Run/Ren* (for powdery mildew caused by *Erysiphe*/*Uncinula necator*) loci. To date 31 *Rpv* loci originating from different genetic sources have been described. Vitis International Variety Catalog (VIVC; Maul 2023) has published a list of all *Rpv* and *Run/Ren* loci with additional information. The most recently discovered *Rpv* loci – *Rpv29*, *Rpv30* and *Rpv31* have been found directly in *V. vinifera* L. and published by (Sargolzaei et al. 2020). Regarding *Run/Ren*, 14 loci have been found and also published in the VIVC list until today. The most recent *Ren11* has been published in Karn et al. (2021).

The functional mechanisms of individual loci reaction can differ and is probably determined by the botanical origin of their bearers. Some of these loci, *e.g.*: *Rpv3* (Bellin et al. 2009) can cause local hypersensitive reaction and can completely stop the pathogen infection, similarly as *Rpv1* (Feechan et al. 2013). On the other hand, some of the loci have been described as weak, but it has been proved that these minor loci have cumulative effect and work closely with major loci and significantly increase the resistance response (Schwander et al. 2012; Agurto et al. 2017; Saifert et al. 2018). Growing grapevine varieties which carry at least one resistant loci with a strong effect can provide a significant decrease of environment contamination by chemical products that are used for vineyards protection every year. Genotypes that have more resistance genes in their genome are much more advantageous in terms of sustaining long-lasting resistance. As described in Eibach et al. (2007), the use of simple sequence repeat (SSR) markers linked to respective loci is a fast and effective way to evaluate the profile of present resistance loci in the analysed genotype. Aim of our work was to analyse 19 interspecific and 1 conventional grapevine cultivar (non-resistant control) for 6 different resistance loci. We were working with 10 relatively new genotypes bred in Czech Republic, 5 of them have not been registered yet. Other interspecific genotypes, we were working with, are commonly grown in international vineyards. For most of these genotypes, their pedigree is known, but the resistance loci actually represented in their genome are often not known. To fill this gap, the main aim of this work was to analyse these genotypes in terms of harboured loci of resistance and evaluate their further breeding potential, especially from the point of view of pyramiding for long-lasting resistance.

Almost every variety has a known pedigree, so there is a theoretical guess, which resistance locus can appear in their genome. However, pedigree of Peking has not yet been confirmed and breeders can only assume that this cultivar originates from the genus *V. amurensis* Rupr., however, as this cultivar originates from a botanical garden where pollination by other vine species may have occurred, its genetic background may be far more complex.

MATERIAL AND METHODS

Plant material. Totally 20 grapevine cultivars (Table 1) were included in the study. Analysed genotypes originate from the gene pools managed at the Faculty of Horticulture Mendel University in Brno and from the vineyards of grape breeding company VINSELEKT MICHLOVSKÝ a.s.

DNA extraction. DNA was extracted from leaves with the DNeasy Plant Mini Kit (Qiagen, Netherlands) according to the manufacturer's protocol. The concentration of each DNA solution was measured using Quant-iT™ PicoGreen™ Kit (ThermoFisher, USA).

***R*-loci analysis.** For each analysed *R*-locus (resistance locus) a specific linked SSR marker was used based on a previous reference study (Table 2).

It is a general feature of capillary electrophoresis that the measured allele size may vary slightly between individual laboratories (This et al. 2004). Thus comparison of absolute allele sizes with other studies is problematic. To deal with this problem, a reference genotype for each controlled loci of resistance was chosen. The reference genotypes were also generally selected to have the highest possible similarity with the pedigrees of the analysed cultivars in terms of their pedigree of resistance sources. Namely cv. Regent was used as a reference for *Rpv4* locus (Welter et al. 2007), *Ren3* locus (van Heerden et al. 2014) and *Ren9* locus (Zendler et al. 2017), cv. Villard Blanc for *Rpv3* locus (Di Gaspero et al. 2012), cv. Bianca for *Rpv7* locus (Bellin et al. 2009) and cv. Solaris for *Rpv10* locus (Schwander et al. 2012). Pure *V. vinifera* L. cultivar Pálava was used as a non-resistant control.

The PCR reaction was performed in a volume of 20 µL. Mastermix contained G2 Flexi buffer 1×, 1.5 mM $MgCl_2$, 0.1 mM dNTP, 0.2 µM of each primer, 0.75 U G2 Flexi Go*Taq* polymerase (Promega, USA), 3 ng of DNA. Amplification was performed

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Table 1. Genotypes used for analysis

Cultivar	Country of origin	Crossing	Additional information
Bianca	HU	Villard Blanc × Bouvier	
Cabernet Cortis	GER	Cabernet Sauvignon × Solaris	
Calardis Blanc	GER	Calardis Musque × Seyve Villard 39-639	
Muscaris	GER	Solaris × Muscat à Petits Grains Blancs	
Peking	–	–	https://vivc.de
Regent	GER	Diana × Chambourcin	
Sauvignac	SWI	(Sauvignon × Riesling) × ?	
Solaris	GER	Merzling × Geisenheim 6493	
Villard Blanc	FR	Seibel 6468 × Subereux	
Erilon	CZE	(Blaufränkisch × Cabernet Franc) × (Merlot × Seibel 13666)	
Kofranka	CZE	(Merlot × Seibel 13666) × (Blaufränkisch × Saint Laurent)	
Malverina	CZE	Rakish × Merlan	
Marcus Blanc	CZE	–	
Pálava	CZE	Traminer × Müller Thurgau	
Pinot Écru	CZE	–	ESM
Riesling Gris	CZE	–	
Rinot	CZE	Merzling × (Seyve Villard 12375 × Pinot Gris)	
Ruby Pinot	CZE	–	
Runa	CZE	–	
Savilon	CZE	Rakish × Merlan	

GER – Germany; CZE – Czech Republic; SWI – Switzerland; FR – France; HU – Hungary; genotypes without crossing are being in the registration process and their pedigree cannot be published – for photos and brief description see the Electronic Supplementary Material (ESM)

Table 2. Markers used in analysis with additional information

Loci	Marker		Sequence 5'→3'	Annealing temperature (°C)	References
Rpv3	UDV737	F R	TTTGCATGCGATACCTGAAG TCCTGCAGCTGTTGACGATA	47	Di Gaspero et al. (2012)
Rpv4	VMC7h3	F R	TCAGATATTGAAGAACACCACA ACTAGAAAATGCACAATCTCCC	51	Welter et al. (2007)
Rpv7	UDV097	F R	ACTTACACTTTCTTTTGTACATGTGAG TGACTGCAAAAACCCTAACAGA	56	Bellin et al. (2009)
Rpv10	GF09-44	F R	CATCGTTCCTTTCTTACTCGCT GCTAATGGAGGGTAGTGCTCAA	53	Schwander et al. (2012)
	GF09-55	F R	GAAATGGGAGGAGTTTTCACA AGTGCTCTTTCCTAGTGGATG	47	Schwander et al. (2012)
Ren3	ScORGF15-02	F R	TCAGAAAAATTGCTGTGATATG GCCCATAAGGTAAAATCCCTTC	47	Zendler et al. (2017)
Ren9	CenGen6	F R	TGGTCAATGATCTCCCCATT TTCCAATCAAGGTCATGCAA	55	Zendler et al. (2020)

F – forward; R – reverse

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in MJ thermocycler as follows: initial denaturation (95 °C, 5 min), then 35 cycles of denaturation (95 °C, 30 s), annealing (47–56 °C, 30 s; temperature was optimized for each primer pair), elongation (72 °C, 30 s) and then final elongation (72 °C 5 min). SSR products were analysed as in Baránková et al. (2020). Briefly, PCR products were separated using a genetic analyser (ABI Prism 310; Applied Biosystems, Carlsbad, USA), and the size of the alleles was determined by GeneScan software (Ver. 3.7, Applied Biosystems, USA). The presence of resistance loci in the analysed genotype was confirmed on the basis of shared allele with reference cultivar.

RESULTS

Cv. Regent was used as a reference for *Ren3*, *Ren9* and *Rpv4* loci. Lengths of the fragments which were gained using respective linked SSR loci i.e. ScORGF15-02 (*Ren3*), CenGen6 (*Ren9*) and VMC7h3 (*Rpv4*) were compared with lengths presented in original studies. Based on this comparison the candidate allele with precise length was determined as 120 bp for *Rpv4* locus, 242 bp for *Ren3* and 284 bp for *Ren9* locus. Subsequently, its presence was evaluated for the rest of the analysed varieties. Similarly, Bianca was used as a reference for *Rpv7* locus using the UDV097 marker. Results were compared with the original study and the candidate allele of length 222 bp indicating the presence of *Rpv7* locus was also found (Table 3).

Resistance locus *Rpv3* was characterised using the UDV737 marker and applying Villard Blanc and Seibel 4614 as references and 287 and 279 bp amplicons as *Rpv3* indicating alleles. The presence of *Rpv10* resistance was deduced based on the presence of candidate GF-09-44 marker allele (231 bp) identified by analysis of the reference cv. Solaris.

With this SSR marker, a problem with the possible coincidence of the identification allele even in non-resistant genotypes (see discussion for more) was revealed, so the presence of the *Rpv10* locus was confirmed by the SSR marker GF-09-55, which is also linked to it.

Based on the above described principle of indicating allele determination all analysed cultivars were finally assessed on the presence of 6 important loci of resistance against downy and powdery mildew. Obtained information is collected within Table 3 and discussed below.

From the results presented, it is clear that the *Rpv3* locus was present in all tested interspecific genotypes and therefore has the most numerous representation in the monitored set of genotypes. The second most frequent was the *Rpv7* locus found in 13 varieties. The occurrence of tested loci responsible for powdery mildew resistance was also relatively frequent, *i.e.* loci *Ren3* and *Ren9*. On the contrary, the *Rpv10* and *Rpv4* loci were represented relatively little, while in the case of *Rpv10* this may be due to the fact that the relevant source of resistance derived from *V. amurensis* Rupr. is used in breeding for a relatively short time. From the point of view of the pyramiding of resistance loci, we recorded 5 harboured *R*-loci in 4 analysed international genotypes, namely Villard Blanc, Bianca, Regent and Cabernet Cortis. The same number was confirmed in the relatively recently bred variety Rinot. Additional information obtained from our analysis is described in more detail below in the discussion.

DISCUSSION

The Czech Republic is one of the wine regions where the popularity of interspecific varieties is gradually increasing because of their resistance properties and thus less chemical sprays during the growing season. For example, the area of Pilzwiderstandsfähige Rebsorten (PIWI) varieties grown in Czech Republic has multiplied 20 times since 2007 and at the end of 2022 were these varieties grown in an area of 777 hectares. It is therefore evident that with the provided economic and environmental benefits, these genotypes are able to provide top quality and competitive wines compared to their well known *V. vinifer*a L. siblings. Except for internationally used *V. vinifera* L. cultivars, new Czech interspecific varieties were registered for planting by state authorities in the last two decades. It is surprising that, despite their use, background of their resistant phenotype was described only on the parental pedigrees and field observations.

The most difficult interpretation was for *Rpv3* loci analysis, as it's generally described as a very complex loci localized on chromosome 18. Zini et al. (2019) claims that it is the most commonly used loci across breeding of interspecific genotypes. Its strength is considered as partial, but many studies such as Schwander et al. (2012) or Merdinoglu et al. (2003) proved that other loci increase strength of *Rpv3* when combined in one genotype. Di Gaspero et al. (2012) have deeply analysed the *Rpv3* through its ancestors' lines and discovered even 7 differ-

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Table 3. Resistance loci analysis results

Name		*Rpv3*		*Rpv4*		*Rpv7*		*Rpv10*				*Ren3*		*Ren9*	
Marker	VIVC No.	UDV737	UDV737	VMC7h3		UDV097		GF-09-44		GF-09-55		ScORGF15-02		CenGen6	
RG		Villard Blanc	Seibel 4614	Regent		Bianca		Solaris		Solaris		Regent		Regent	
RGS		287	279	120		222		231		249		242		284	
Bianca	1321	**287**	302	**120**	130	**222**	194	235	212	221	247	228	**242**	263	**284**
Cabernet Cortis	20005	210	**279**	148	130	163	**222**	**231**	238	**249**	254	239	**242**	270	**284**
Calardis Blanc	22828	**287**	304	130	148	**222**	163	*231*	242	220	254	**242**	228	269	266
Muscaris	22628	**287**	240	130	146	108	194	**231**	243	**249**	246	**242**	228	268	272
Peking	15077	**287**	306	142	130	**222**	224	**231**	238	**249**	254	238	**242**	266	270
Regent	4572	**287**	304	**120**	130	194	**222**	236	242	245	253	228	**242**	272	**284**
Sauvignac	22322	**287**	330	130	142	185	190	212	240	235	247	**242**	226	263	260
Solaris	20340	250	**279**	148	132	164	220	228	**231**	245	**249**	**242**	**242**	**284**	190
Villard Blanc	13081	**287**	308	**120**	130	**222**	194	240	260	236	240	228	**242**	270	**284**
Erilon	17509	**287**	301	136	130	108	**222**	242	243	220	245	**242**	226	**284**	270
Kofranka	17535	**287**	301	130	140	**222**	105	236	236	220	254	228	**242**	263	**284**
Malverina	17523	**287**	298	146	148	**222**	160	240	240	236	246	**242**	228	270	**284**
Marcus Blanc	–	**287**	276	114	130	164	248	240	242	236	246	**242**	**242**	272	**284**
Pálava	8875	224	332	130	145	184	172	*231*	245	235	254	207	231	274	196
Pinot Ecru	–	**287**	271	136	130	164	**222**	234	234	236	242	**242**	**242**	272	**284**
Riesling Gris	–	**287**	294	116	130	108	164	240	240	236	246	**242**	**242**	**284**	**284**
Rinot	23932	**287**	294	**120**	130	164	**222**	240	242	236	246	136	**242**	272	**284**
Ruby Pinot	–	**287**	306	130	146	162	**222**	242	242	246	254	**242**	**242**	268	272
Runa	–	**286**	298	146	148	128	164	216	240	236	246	**240**	**240**	**284**	**284**
Savilon	22823	**287**	276	132	141	**222**	246	234	240	236	246	**242**	**242**	272	**284**

Numbers in the table mean basepair; bold – presence of resistance loci; RG – reference genotype; RGS – reference genotype size; VIVC – the Vitis International Variety Catalogue

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ent haplotypes of *Rpv3*. We have chosen UDV737 (Di Gaspero et al. 2012) marker for our analysis because of its universality for each of 7 haplotypes. *Rpv3* loci were confirmed in every genotype that we have analysed. We have found two different alleles signalling presence of this locus – 287 bp and 279 bp. Interesting point is, that we have found identification allele 287 bp also in cv. Peking. Unlike the other analysed genotypes, pedigree of Peking had not yet been confirmed and breeders can only assume that this cultivar belongs to the genus *V. amurensis* Rupr. In view of this fact, a few theoretical explanations are offered for this unexpected record of an allele of 287 bp length also in the Peking genotype initially used as a pure representative of the genus *V. amurensis* Rupr. A more in-depth subsequent analysis of this observation would be needed for a definitive answer. Generally, it is possible to state that many haplotypes of *Rpv3* locus and thus different sized alleles with UDV737 marker can be expected, which makes their evaluation quite difficult. A possible measure for similar analysis is to use or combine with another markers linked to *Rpv3* as GF-18-06 and GF-18-08 that were published in Zyprian et al. (2016) as these two markers were reported as providing only one allele size.

Locus *Rpv4* localized on chromosome 4 has been confirmed in Regent cultivar with 122 bp allele as a signal of presence. Strength of the *Rpv4* is considered as minor, but Welter et al. (2007) claims that this locus closely cooperates with *Rpv3* locus and increases its resistance response. *V. rupestris* Scheele is considered as an original species. We have confirmed this locus in 4 cultivars, namely Villard Blanc, Regent, Bianca and Czech variety Rinot.

Regarding *Rpv7*, Marguerit et al. (2009) claim that this minor locus originates from the *V. riparia* Michx. However Bellin et al. (2009) have discovered this locus in Bianca cultivar with 222 bp allele and claim that its origin is connected with many American *Vitis* L. species. Our study confirmed this locus in 13 genotypes with many American *Vitis* L. species in their pedigree, *e.g.*: *V. riparia* Michx., *V. rupestris* Scheele or *V. Berlandieri* Planch. This locus was also found in Peking. The comment in this case is similar to that for the *Rpv3* locus.

Schwander et al. (2012) have proved that *Rpv10* locus has its origin in the Asian species *V. amurensis* Rupr. Regarding the breeding strategies, cv. Severnyj was the mainly used genotype, however its offspring Cvetocnyj has been frequently used for crossing too. These days we have many new interspecific varieties with *V. amurensis* Rupr. in their genetic profile, such as the well known Solaris which was used for *Rpv10* analysis in Schwander et al. (2012) study. With this in mind, GF-09-44 marker with cv. Solaris as a reference genotype has been initially chosen to establish candidate allele indicating the presence of *Rpv10* locus. As doing so, it was evident that every genotype with *V. amurensis* Rupr. in their pedigree amplified the 231 bp allele. However, we have also found this allele in Calardis Blanc and Pálava, while none of these genotypes have no *V. amurensis* Rupr. in its pedigree. We have decided to use another marker GF-09-55 linked to *Rpv10* as a confirmation of the obtained results. Every genotype with *V. amurensis* Rupr. in their pedigree gave us then 231 bp allele for GF-09-44 marker and 249 bp allele for GF-09-55 marker. But with marker GF-09-55 Calardis Blanc produced 220 and 254 bp alleles and Pálava produced 235 and 254 bp alleles. Interestingly, joint property of these two varieties is that they have Traminer genotype in their pedigree. To verify this hypothesis, additional analysis of Savagnin blanc as a typical representative of sensitive and Traminer harbouring cultivar was performed. Interestingly, this cultivar by analysis of GF-09-44 marker produced a 231 bp long allele and 235 and 255 bp alleles for GF-09-55 marker. This suggests that the use of GF-09-44 marker in breeding and selection processes is problematic and essentially impossible for genotypes that have Traminer in their pedigree.

The *Ren3* locus has been initially found on the basis of genetic mapping by using Regent offsprings Shidfar et al. (2019). The specific *Vitis* L. species, which could be the original source of *Ren3* has never been found yet, however Zendler et al. (2017) claim that most likely species are *V. lincecumii* Buckl. and *V. rupestris* Scheele. Each genotype in our experiment that contains at least one of these species in its genetic pedigree, harbours an allele of size 242 bp as a sign of complex and probably multi-sourced loci *Ren3*. Interestingly, this locus was also found in Peking as a representative of *V. amurensis* Rupr. As mentioned in van Heerden et al. (2014) study, Regent has a lineage composed of 8 *Vitis* L. species, except *V. amurensis* Rupr. This fact greatly contributes to the hypothesis of a complex genetic background of this variety.

Locus *Ren9* has been initially considered as a part of *Ren3*. Based on of Zendler et al. (2020) study during more thorough analysis of *Ren3* suggested

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separating this locus into two parts. The origin of this locus is also attributed to many American species. We have also used Regent as a reference genotype and 287 bp allele was found as a signal of presence for this locus.

CONCLUSION

In the presented work, some internationally recognized varieties as well as some newly registered varieties in the Czech Republic were analysed from the point of view of the presence of resistance loci. Based on these analyses we were able to identify the genotypes with an above-number of pyramidised resistance loci. These genotypes have a higher potential to provide a more stable phenotype of resistance to powdery and downy mildew in the case of new occurence of pathogen strains that would overcome resistance ensured by one locus. Namely, we have identified 4 genotypes harboring 5 resistance loci: Villard Blanc, Bianca, Regent, Cabernet Cortis. These results explain the fact why these genotypes are widely used for breeding. In the view of Czech-bred genotypes with the highest number of harboured resistant loci, the best white-grape genotype is Rinot with 5 resistance loci and blue-grape genotype is Kofranka with 4 loci. We have also discovered the false-positive results problem with marker GF-09-44 in the case that Traminer variety is present in the pedigree.

Overall, this study provides valuable information both for winemakers when selecting genotypes for their future growing, for breeders in terms of selecting suitable parental genotypes for breeding and also for scientists when selecting suitable tests based on SSR markers for laboratory analyses of progeny.

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