

Dissecting the susceptibility/resistance mechanism of *Vitis vinifera* for the future control of downy mildew

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Abstract. The Eurasian grapevine (*Vitis vinifera*), a species cultivated worldwide for high-quality wine production, is extremely susceptible to the agent of downy mildew, *Plasmopara viticola*. Nevertheless, germplasm from Georgia (Southern Caucasus, the first grapevine domestication centre), characterized by a high genetic variability, showed resistance traits to *P. viticola*. The cultivar Mgaloblishvili exhibited the most promising phenotype in terms of resistance against *P. viticola*. Its defence response results in: i) low disease intensity; ii) low sporulation; iii) damaged mycelium; iv) production of antimicrobial compounds such as volatile organic compounds (VOCs), whose effectiveness on the pathogen was evaluated by leaf-disc assays. At the transcriptomic level, its resistance mechanism is determined by the differential expression of both resistance and susceptible genes. The resistance genes are related to: i) pathogen recognition through PAMP, DAMP and effector receptors; ii) ethylene signalling pathway; iii) synthesis of antimicrobial compounds (VOCs) and fungal wall degrading enzymes; iv) development of structural barriers (cell wall reinforcement). The first putative susceptible gene was the transcription factor *VviLBD1f7* gene, whose validation was carried out by dsRNA (double-stranded RNA) assay. In this work, these unique results on plant-pathogen interaction are reviewed with the aim of developing new strategies to control the disease.

1 Georgian germplasm, not only the first grapevine domestication centre

Archaeological and palaeobotanical findings pointed out the Caucasus as the first grapevine (*Vitis vinifera*) domestication centre [1]. This evidence has been confirmed by genetic diversity studies. The genetic characterization of Georgian germplasm was performed by both SSR (simple sequence repeat) [2–4] and SNP (single nucleotide polymorphism) [5–7] molecular markers. Both molecular markers showed high levels of genetic diversity and heterozygosity, attesting the uniqueness and originality of this germplasm in comparison with the European and Central Asian germplasms [3,8–11]. Does this genetic diversity reflect a diversity at the phenotypic level? Do Georgian cultivars show resistance to pathogens, such as *Plasmopara viticola*?

P. viticola is the causal agent of grapevine downy mildew, one of the most important diseases at the

international level. *P. viticola*, native of Northern America, was fortuitously introduced in France during the nineteenth century and rapidly spread across Europe [12]. The pathogen infects all green parts of the plant causing extensive losses in grape yield [13]. European *V. vinifera* cultivars are generally considered susceptible to *P. viticola*. Nevertheless, little information is available on the less common germplasm, such as the Georgian germplasm. Phenotypic investigations aimed to identify possible sources of resistance to *P. viticola* were carried out on Georgian cultivars. The estimation of resistance levels to *P. viticola* by both experimental inoculations and disease assessment in field conditions, allowed us to identify cultivars responding to infection with a lower disease intensity [14,15]. One of these is the cultivar called Mgaloblishvili. Mgaloblishvili is a local blackberry variety from Imereti province in the Western Georgia (Fig. 1). Mgaloblishvili showed a constant resistant behaviour, by reducing the disease severity and

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the pathogen sporulation (Fig. 2), with susceptibility levels lower than 25% [14,16].

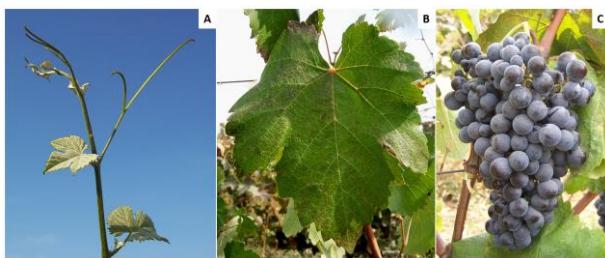


Fig. 1. Mgaloblishvili. A: tip. B: mature leave. C: bunch.

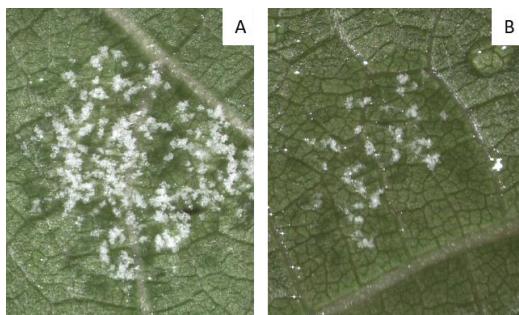


Fig. 2. *P. viticola* sporulation on the susceptible cultivar Pinot noir (A) and on the resistant cultivar Mgaloblishvili (B). A reduced sporulation density is visible on Mgaloblishvili.

2 How does Mgaloblishvili counteract *P. viticola*?

In Mgaloblishvili, confocal microscopy detected a normal colonization of leaf tissues by *P. viticola* up to 2 days after inoculation (dai), where the asexual spores of the pathogen penetrated through the stomatal pore differentiating the substomatal vesicle, the primary hypha and the first haustorium. At 3 dai, *P. viticola* mycelium showed evident alterations: hyphae were hyper-branched, contorted and ill defined. At 6 dai, dead portions of mycelium, surrounded by callose barriers were visible, as well as, short, hyperbranched and partly sterile sporangiophores emerging from the stomata [16].

At the transcriptomic levels, the resistance mechanism results in the overexpression of resistance genes involved in the plant defence pathway and related to: i) pathogen recognition through PAMP (pathogen-associated molecular patterns), DAMP (damage-associated molecular patterns) and effector receptors; ii) phytohormone signalling based on ethylene; iii) resistance response based on synthesis of antimicrobial compounds and fungal wall degrading enzymes; iv) cell wall reinforcement. For each step of plant defence pathway, putative resistance genes were identified. The rust resistance kinase Lr10 (a specific receptor able to recognize the effectors and ultimately to activate the defence mechanism) was involved in recognition, the ethylene-responsive transcription factor in signal transduction, and the endo-1,3;1,4- β -D-glucanase (a PR protein shown to be ethylene-responsive) and the valencene synthase (a sesquiterpene synthase) connected with the resistance response [16]. Future functional

characterization studies will allow us to depict their role in the plant-pathogen interaction.

Besides grapevine genes, the genes overexpressed by the pathogen during the interaction with the plant were investigated. Multiple effector-encoding genes were identified in *P. viticola* transcriptome during the infection of Mgaloblishvili, with remarkable expression differences with the susceptible *V. vinifera* cultivar Pinot noir. Five apoplastic effector genes, putatively encoding for serine proteases involved in protein hydrolysis, specifically associated with resistance in Mgaloblishvili [17].

3 The synthesis of VOCs to counteract the pathogen infection

Volatile organic compounds (VOCs), such as terpenes, norisoprenoids, alcohols and aldehydes, are frequently emitted by plants in response to attack by pathogens [18]. This is also true for grapevine during *P. viticola* infection [16,19]. Transcriptional analysis revealed an overexpression of genes related to the biosynthesis of VOCs, such as valencene synthase and several cytochrome P450s, in Mgaloblishvili leaves inoculated with *P. viticola* [16]. Solid-phase microextraction gas chromatography-mass spectrometry analysis and the expression of terpene synthases ((E)- β -caryophyllene synthase, (E)- α -bergamotene synthase, (E, E)- α -farnesene synthase, (E)- β -ocimene synthase, α -terpineol synthase and valencene synthase), revealed the biosynthesis of VOC in Mgaloblishvili leaves inoculated with *P. viticola*. After pathogen inoculation, the expression pattern of six terpene synthases increased rapidly (at 1 dai) and a constant increment of nine VOCs was observed from 1 dai to 3 dai, supporting the role of VOCs into defence mechanism of Mgaloblishvili against *P. viticola*. Interesting, four (farnesene, nerolidol, ocimene and valencene) out of nine VOCs caused a significant reduction (53–100%) in *P. viticola* sporulation in leaf-disc assays, suggesting their potential role as a natural and eco-friendly solution for a sustainable protection of grapevine from *P. viticola* [20].

4 New *Rpv* loci for the resistance to *P. viticola*

So far, the investigation of the genetic basis of *P. viticola* resistance has led to the identification of 28 resistance loci in different regions of grapevine genome. These loci (designated *Rpv* = Resistance *P. viticola*), identified through QTL (Quantitative Trait Loci) analysis on a range of North American and Asian *Vitis* (non-*vinifera*) species, confer different degrees of disease resistance, ranging from partial to total resistance [21].

Recently, three new loci (*Rpv29*, *Rpv30* and *Rpv31*) associated with resistance to *P. viticola* have been identified in the *vinifera* germplasm coming from the Caucasus, using a GWA (Genome Wide Association) approach (Fig. 3). These new loci have been associated with a low level of pathogen sporulation [22]. *Rpv29*

locus, located on chromosome 14, was mapped in the coding region of HEAT repeat-containing 5B protein, which is a required plant immunity protein. The other characterized genes in the surrounding regions are a probable cellulose synthase A catalytic subunit 8 [UDP-forming], a acyl-CoA-binding domain-containing protein 3-like, a probable carboxylesterase 17 and a plant cadmium resistance 4 protein, which are genes involved in stress signaling and PAMP resistance [22]. The *Rpv30* locus, located on chromosome 3, was in close distance to a MADS-box protein JOINTLESS-like, an ubiquitin carboxyl-terminal hydrolase 21, two magnesium-dependent phosphatases (MDP-1 and MDP-1-like), proteins with plant defensive role [22]. The *Rpv31* locus was annotated in the linkage group of several rust resistance kinase Lr10-like genes on chromosome 16, associated with the *P. viticola* resistance trait in Mgaloblishvili [16]. Loci associated with the resistant traits are very useful in the breeding programs assisted by molecular markers [23]. Genes associated with the *Rpv29*, *Rpv30* and *Rpv31* loci are potential target genes for breeding of *P. viticola* resistant grapevine cultivars.

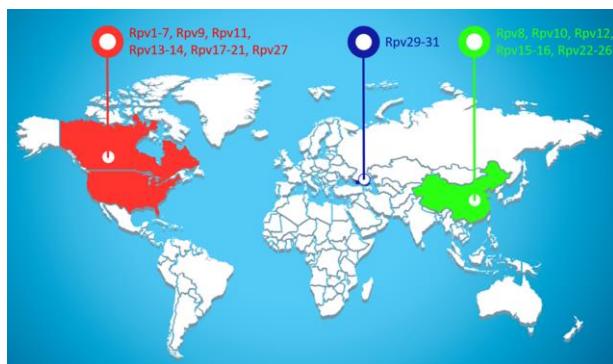


Fig. 3. Map of the origin of *Rpv* (Resistance *P. viticola*) loci. In red, loci identified in North American *Vitis* species. In blue: loci identified in Georgian germplasm (*V. vinifera*). In green: loci identified in Asian *Vitis* species.

5 Besides resistance genes: the first putative susceptible gene

Unfortunately, the resistance mechanisms based on single R (resistance) genes can be overcome due to the high adaptability of the pathogens. Indeed, *P. viticola* isolates that specifically overcome *Rpv3*, the major resistance gene carried by Bianca at chromosome 18, have been already detected [24,25]. A point mutation in a gene encoding for a protein or effector may be enough to escape recognition [26]. To solve this inconvenience, resistance genes pyramiding has to be performed. A different strategy to obtain resistant cultivars can be achieved by exploiting S (susceptibility) genes [26]. Susceptibility genes are genes that facilitate the compatibility between plant and pathogen and are essential for their interaction, especially in the case of biotrophic pathogens, such as *P. viticola*. Mutation or loss of an S gene can limit the ability of the pathogen to cause disease.

Up to now, the only S genes known for grapevine are the MLO genes, whose knockdown confers resistance to the powdery mildew agent, reducing the disease severity up to 77% [27]. Recently, a candidate S gene for *P. viticola* resistance has been suggested. This gene, named *VviLBDIf7*, was found to be overexpressed in the susceptible cultivar Pinot noir 24 hours after the inoculation with *P. viticola*, while its expression was found to be decreased in the resistant Mgaloblishvili cultivar [17]. Moreover, its orthologous in *Arabidopsis thaliana* has been proven to confer resistance to *Fusarium oxysporum* when knockout [28]. *VviLBDIf7* gene is a transcription factor belonging to a family of LOB (LATERAL ORGAN BOUNDARIES) transcription factor, which are involved in the regulation of plant organ development and in the response to abiotic and biotic stresses [29]. The inactivation of *VviLBDIf7* in *V. vinifera* could give proof of its involvement in *P. viticola* resistance.

RNA interference (RNAi) by exogenous double-stranded RNA (dsRNA) is a transient way to knockdown target genes [30]. This strategy has been used to knockdown the *VviLBDIf7* gene in a highly susceptible grapevine cultivar (Pinot noir) to control *P. viticola* infections. Exogenous application of dsRNA led to significant reductions in both *VviLBDIf7* gene expression and disease severity. In addition, clear alterations to both vegetative (hyphae and haustoria) and reproductive structures (sporangiphores) have been identified on leaves treated with dsRNA, resulting in a stunted growth and reduced sporulation [31]. These observations prove that *VviLBDIf7* gene is a candidate gene to be silenced to reduce susceptibility to *P. viticola*.

6 Conclusions

The damages caused by *P. viticola*, combined with the ineffectiveness of agronomic practices in containing its diffusion, oblige frequent application of fungicides, making viticulture as the agricultural activity using the most intensive amount of plant protection products. The repeated use of fungicides impacts negatively on farm budgets and on human health and environment, and leads to the selection of resistance to fungicides. Moreover, the Directive 2009/128/EC and Regulation (EC) No. 1107/2009 of the European Parliament and of the Council concerning the placing and use of plant protection products on the market imposes to the farmers to reduce the use of agrochemicals during the next years. For these reasons, the cultivation of pathogen-resistant grapevine varieties is one of the most immediate strategies to reduce the impact of plant protection and ensure, at the same time, quantity and quality of yield.

If a few years ago resistance to *P. viticola* in *V. vinifera* was a utopia, today it is a certainty. Indeed, several Georgian cultivars showing resistance traits to *P. viticola* were identified and their study allowed us to identify putative resistance and susceptibility genes. These genes pave the way for the future of breeding programs, that can be based on innovative breeding technologies, such as the genome editing, or on MAS

(Marker Assisted Selection), once the inheritance of the trait has been assessed. Finally, the identification of terpenes able to reduce downy mildew severity, as well as the ability of dsRNA to reduce pathogen infection, can be exploited for the development of new eco-friendly solutions to protect grapevine from *P. viticola* attack.

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References

1. H. P. Olmo, P. E. McGovern, S. J. Fleming, and S. H. Katz, McGovern P, Éd. Orig. Anc. Hist. Wine. Amsterdam Gordon Breach **31**, 43 (1995)
2. V. Laucou, T. Lacombe, F. Dechesne, R. Siret, J.-P. Bruno, M. Dessup, T. Dessup, P. Ortigosa, P. Parra, C. Roux, S. Santoni, D. Varès, J.-P. Péros, J.-M. Boursiquot, and P. This, Theor. Appl. Genet. **122**, 1233 (2011)
3. S. Imazio, D. Maghradze, G. Lorenzis, R. Bacilieri, V. Laucou, P. This, A. Scienza, and O. Failla, Tree Genet. Genomes **9**, 641 (2013)
4. J. Ekhvaia, M. Gurushidze, F. R. Blattner, and M. Akhalkatsi, Genet. Resour. Crop Evol. **61**, 1507 (2014)
5. G. De Lorenzis, R. Chipashvili, O. Failla, and D. Maghradze, BMC Plant Biol. **15**, 154 (2015)
6. V. Laucou, A. Launay, R. Bacilieri, T. Lacombe, A.-F. Adam-Blondon, A. Bérard, A. Chauveau, M. T. de Andrés, L. Hausmann, J. Ibáñez, M.-C. Le Paslier, D. Maghradze, J. M. Martinez-Zapater, E. Maul, M. Ponnaiah, R. Töpfer, J.-P. Péros, and J.-M. Boursiquot, PLoS One **13**, 1 (2018)
7. F. Mercati, G. De Lorenzis, A. Mauceri, M. Zerbo, L. Brancadoro, C. D’Onofrio, C. Morcia, M. G. Barbagallo, C. Bignami, M. Gardiman, L. de Palma, P. Ruffa, V. Novello, M. Crespan, and F. Sunseri, Front. Plant Sci. **12**, (2021)
8. S. Myles, A. R. Boyko, C. L. Owens, P. J. Brown, F. Grassi, M. K. Aradhya, B. Prins, A. Reynolds, J.-M. Chia, D. Ware, C. D. Bustamante, and E. S. Buckler, Proc. Natl. Acad. Sci. U. S. A. **108**, 3530 (2011)
9. R. Bacilieri, T. Lacombe, L. Le Cunff, M. Di Vecchi-Staraz, V. Laucou, B. Genna, J.-P. Péros, P. This, and J.-M. Boursiquot, BMC Plant Biol. **13**, 25 (2013)
10. S. Riaz, G. De Lorenzis, D. Velasco, A. Koehmstedt, D. Maghradze, Z. Bobokashvili, M. Musayev, G. Zdunic, V. Laucou, M. Andrew Walker, O. Failla, J. E. Preece, M. Aradhya, and R. Arroyo-Garcia, BMC Plant Biol. **18**, 137 (2018)
11. G. De Lorenzis, F. Mercati, C. Bergamini, M. F. Cardone, A. Lupini, A. Mauceri, A. R. Caputo, L. Abbate, M. G. Barbagallo, D. Antonacci, F. Sunseri, and L. Brancadoro, BMC Plant Biol. **19**, 7 (2019)
12. M. C. Fontaine, F. Austerlitz, T. Giraud, F. Labbé, D. Papura, S. Richard-Cervera, and F. Delmotte, Mol. Ecol. **22**, 2771 (2013)
13. Y. Yu, Y. Zhang, L. Yin, and J. Lu, Phytopathology **102**, 1094 (2012)
14. S. L. Toffolatti, G. Maddalena, D. Salomoni, D. Maghradze, P. A. Bianco, and O. Failla, Vitis - J. Grapevine Res. **55**, 121 (2016)
15. N. Bitsadze, M. Aznarashvili, A. Vercesi, R. Chipashvili, O. Failla, and D. Maghradze, Vitis - J. Grapevine Res. **54**, 193 (2015)
16. S. L. Toffolatti, G. De Lorenzis, A. Costa, G. Maddalena, A. Passera, M. C. Bonza, M. Pindo, E. Stefani, A. Cestaro, P. Casati, O. Failla, P. A. Bianco, D. Maghradze, and F. Quaglino, Sci. Rep. **8**, 12523 (2018)
17. S. L. Toffolatti, G. De Lorenzis, M. Brilli, M. Moser, V. Shariati, E. Tavakol, G. Maddalena, A. Passera, P. Casati, M. Pindo, A. Cestaro, D. Maghradze, O. Failla, P. A. Bianco, and F. Quaglino, Genes (Basel). **11**, 261 (2020)
18. N. Dudareva, F. Negre, D. A. Nagegowda, and I. Orlova, CRC Crit. Rev. Plant Sci. **25**, 417 (2006)
19. V. Lazazzara, C. Bueschl, A. Parich, I. Pertot, R. Schuhmacher, and M. Perazzolli, Sci. Rep. **8**, 1618 (2018)
20. V. Ricciardi, D. Marcianò, M. Sargolzaei, G. Maddalena, D. Maghradze, A. Tirelli, P. Casati, P. A. Bianco, O. Failla, D. Fracassetti, S. L. Toffolatti, and G. De Lorenzis, Plant Physiol. Biochem. **160**, 294 (2021)
21. D. Merdinoglu, C. Schneider, E. Prado, S. Wiedemann-Merdinoglu, and P. Mestre, OENO One **52**, 203 (2018)
22. M. Sargolzaei, G. Maddalena, N. Bitsadze, D. Maghradze, P. A. Bianco, O. Failla, S. L. Toffolatti, and G. De Lorenzis, Front. Plant Sci. **11**, (2020)
23. R. Eibach and R. Töpfer, in *Grapevine Breed. Programs Wine Ind.*, edited by A. Reynolds (Elsevier B.V., 2015), pp. 3–22
24. E. Peressotti, S. Wiedemann-Merdinoglu, F. Delmotte, D. Bellin, G. Di Gaspero, R. Testolin, D. Merdinoglu, and P. Mestre, BMC Plant Biol. **10**, 147 (2010)
25. S. Toffolatti, G. Venturini, D. Maffi, and A. Vercesi, BMC Plant Biol. **12**, 124 (2012)
26. C. C. N. Van Schie and F. L. W. Takken, Annu. Rev. Phytopathol. **52**, 551 (2014)
27. S. Pessina, L. Lenzi, M. Perazzolli, M. Campa, L. Dalla Costa, S. Urso, G. Valè, F. Salamini, R. Velasco, and M. Malnoy, Hortic. Res. **3**, 16016 (2016)
28. L. F. Thatcher, J. J. Powell, E. A. B. Aitken, K.

- Kazan, and J. M. Manners, *Plant Physiol.* **160**, 407 (2012)
29. C. Xu, F. Luo, and F. Hochholdinger, *Trends Plant Sci.* **21**, 159 (2016)
30. A. S. Dubrovina and K. V. Kiselev, *Int. J. Mol. Sci.* **20**, 2282 (2019)
31. D. Marcianò, V. Ricciardi, A. Marone Fassolo, E. Passera, P. Bianco, O. Failla, P. Casati, G. Maddalena, G. De Lorenzis, and S. Toffolatti, *Front. Plant Sci.* (2021)