The *Rpv3-3* haplotype and stilbenoid induction mediate downy mildew resistance in a grapevine interspecific population

S. Vezzulli[a], G. Malacarne[a,b], D. Masuero, A. Vecchione, C. Dolzani, V. Goremykin, Z. Haile Mehari, E. Banchi, R. Velasco, M. Stefanini, U. Vrhovsek, L. Zulini, P. Franceschi and C. Moser

Research and Innovation Centre, Fondazione Edmund Mach, 38010 San Michele all'Adige, Italy.

Abstract

The cultivated Eurasian grapevine (*Vitis vinifera* L.) is highly susceptible to downy mildew (DM) – caused by the biotrophic oomycete *Plasmopara viticola* (Berk. & M.A. Curtis) Berl. & De Toni – the major disease of temperate-humid climates among various pathogen threats. DM control relies mainly on the massive use of fungicides leading to environmental pollution, development of resistance and residual toxicity. The exploitation of DM-resistant wild genetic resources for the development of new resistant cultivars represents a promising alternative. Taking advantage of a segregating population derived from 'Merzling' (M, a mid-resistant hybrid) and 'Teroldego' (T, a susceptible landrace), recent studies have highlighted the importance of stilbenoids among phenolic compounds in conferring resistance to this oomycete. In order to elucidate the genetic bases of DM resistance and polyphenol biosynthesis upon *P. viticola* inoculation, 136 M×T F_1 individuals were characterized by an integrative approach combining genetic, phenotypic and gene-expression data. An improved M×T linkage map was obtained by scoring 192 microsatellite markers. The progeny was further screened for degree of resistance and production of 42 phenolic compounds (including 18 different stilbenoids). Quantitative trait locus (QTL) mapping showed that DM resistance is associated with a specific haplotype at the *Rpv3* locus – herein named *Rpv3-3*, derived from the French hybrid 'Seyval' – and identified 46 novel metabolic (m)QTLs linked to 30 polyphenol-related parameters. A list of the 76 most relevant candidate genes was generated by specifically exploring the genomic regions underlying the mQTLs associated with the stilbenoids induced by the infection. Finally, expression analysis of 13 genes in *Rpv3-3*$^{+/-}$ genotypes, displaying divergent DM resistance and stilbenoid accumulation, revealed significant candidates for the genetic control of stilbenoid biosynthesis and oligomerization. These findings emphasize that DM resistance can be mediated by the major *Rpv3-3* locus and stilbenoid induction.

Keywords: 'Merzling', *Plasmopara viticola*, peroxidase, polyphenols, QTL analysis, secondary metabolites, *Vitis* spp.

INTRODUCTION

All grapevine (*Vitis vinifera* L.) cultivars traditionally grown in Europe are susceptible to downy mildew (DM) caused by *Plasmopara viticola* (Berk. & M.A. Curtis) Berl. & De Toni, an oomycete able to attack any grapevine green tissue. DM control relies mainly on the use of synthetic fungicides, which are costly and have negative environmental impact. The exploitation of *Vitis* genetic resources for the development of new DM-resistant cultivars is a promising alternative.

[a]These authors contributed equally to this study.
[b]E-mail: giulia.malacarne@fmach.it

More than 20 quantitative trait loci (QTLs) associated with DM resistance in different genetic backgrounds are known (VIVC, 2018). The major *Rpv* loci originated from American and Asian *Vitis* species (Merdinoglu et al., 2003; Marguerit et al., 2009; Blasi et al., 2011; Schwander et al., 2012; Ochssner et al., 2016; Divilov et al., 2018). In particular, the *Rpv3* locus is a major determinant of grapevine DM resistance, where seven conserved haplotypes were identified in five descent groups of resistant cultivars and traced back to their founders, which belong to *Vitis rupestris*, *Vitis lincecumii*, *Vitis riparia* and *Vitis labrusca* (Di Gaspero et al., 2012). Until now, only two haplotypes at the *Rpv3* locus have been validated in segregating populations derived from different DM-resistance donors.

The related DM-resistance mechanisms are partially known and are due to gene for gene recognition, signal cascade and defense responses, including the synthesis of secondary metabolites such as stilbenoids. Previous studies conducted at the E. Mach Foundation, taking advantage of a segregating population derived from 'Merzling' (M, a mid-resistant hybrid) and 'Teroldego' (T, a susceptible landrace), pointed to the importance of the stilbenoids in conferring DM resistance (Malacarne et al., 2011). To date, no study has investigated the genetic bases of polyphenol, in particular stilbenoid, synthesis or variation upon *P. viticola* infection. Excluding anthocyanins, most polyphenols do not have a known QTL associated; only a few research studies on proanthocyanidins, or condensed tannins, and flavonol berry composition have recently been attempted in grapevine (Huang et al., 2012; Malacarne et al., 2015).

With the aim of elucidating the genetic bases of DM resistance and polyphenol production upon *P. viticola* inoculation, 136 F_1 individuals were characterized by an integrative approach combining genetic, phenotypic and gene expression data.

MATERIALS AND METHODS

Plant material and phenotyping

In the 2012 growing season, 136 F_1 individuals derived from a cross between the complex *Vitis* hybrid 'Merzling', descended from *V. vinifera*, *V. rupestris* and *V. lincecumii*, and *V. vinifera* 'Teroldego' were grown as potted plants in a greenhouse at 25/20°C day/night temperature, with a 16-h photoperiod and relative humidity of 70±10%. Propagation of the two parental lines was carried out during 2013. All genotypes were *P. viticola*-inoculated on both potted plants (P) and leaf disks (LD) (Vezzulli et al., 2018). *P. viticola* inoculum, named PVL-2012, was derived from a collection on susceptible *V. vinifera* cultivars in an untreated field. The DM response was evaluated at 8 days post-inoculation (dpi) on P and at 4, 5, and 6 dpi on LD by means of three parameters: severity, incidence, and the OIV 452 (for P) or OIV452-1 (for LD) descriptor (OIV, 2009). The second and third leaves from the P apex were collected at 6 dpi (828 samples) and analyzed for the content of 42 phenolics (18 different stilbenoids) by targeted metabolomics (Vrhovsek et al., 2012); an additional 22 sum/ratio parameters were calculated.

Genotyping, QTL analysis and candidate gene selection

The 136 F_1 individuals and the two parental lines were characterized at the genotypic level by means of 192 microsatellite markers. Prior to building the 'Merzling' × 'Teroldego' (M×T) genetic map, simple sequence repeat (SSR) markers were tested against the expected segregation ratio using a χ^2 goodness-of-fit test implemented in JoinMap v.4.1 (JM; Van Ooijen, 2006). Highly distorted ($P>0.05$) markers were discarded, while the others ($P\leq0.05$) were used for linkage analysis unless they affected the order of neighboring loci. Mapping parameters were set at a logarithm-of-odds (LOD) value of 8 and at a recombination frequency of 0.45.

Genetic map data were integrated with phenotypic data and QTL mapping was performed for each experiment separately by using the simple Interval Mapping algorithm in MapQTL v.6.0 (Van Ooijen, 2009). QTLs were declared significant if the maximum LOD exceeded the linkage group (LG)-wide LOD threshold (1000 permutations) and the mean error rate was <0.05. Candidate genes (CGs) included in the confidence interval of reliable

QTLs were selected from the PN40024 12X v.2 reference genome (http://genomes.cribi.unipd.it) based on proximity to LOD peak offset, involvement in trait regulation based on literature, assignment to over-represented functional categories, and involvement in functional categories of interest. Finally, expression analysis of some CGs of interest in 12 F_1 individuals with divergent features was performed by quantitative RT-PCR (data not shown).

Statistical analysis

Statistical analyses applied to phenotypic data were performed in R (R Core Team, 2018) equipped with tidyverse (Wickham, 2017) and ggplot2 (Wickham, 2016) packages.

RESULTS AND DISCUSSION

DM resistance and polyphenol content traits

Of the 192 scored, 181 markers were actually mapped into the M×T linkage map that was built at LOD=8. This genetic tool represents an advanced version in terms of marker number/order and progeny individuals compared with the map of Salmaso et al. (2008). The phenotypic analysis indicated an approximately normal distribution of the DM resistance parameters (Figure 1), and a significant induction of different stilbenoids upon *P. viticola* inoculation; the latter occurred in a subset of F_1 individuals that are characterized by a high degree of resistance. In particular, inoculated samples of resistant individuals showed a significant increase of eight monomers (astringin, *cis*-piceid, *cis*-resveratrol, isorhapontin, piceatannol, pterostilbene, *trans*-piceid, *trans*-resveratrol), five dimers (ampelopsin D + quadrangularin A, *cis*-ε-viniferin, *cis*/*trans* ω-viniferin, pallidol, trans-ε-viniferin), three trimers ((α-viniferin, E-*cis*-miyabenol C,Z-miyabenol C) and two tetramers (ampelopsin H + vaticanol C-like isomer andisohopeaphenol) (Figure 2).

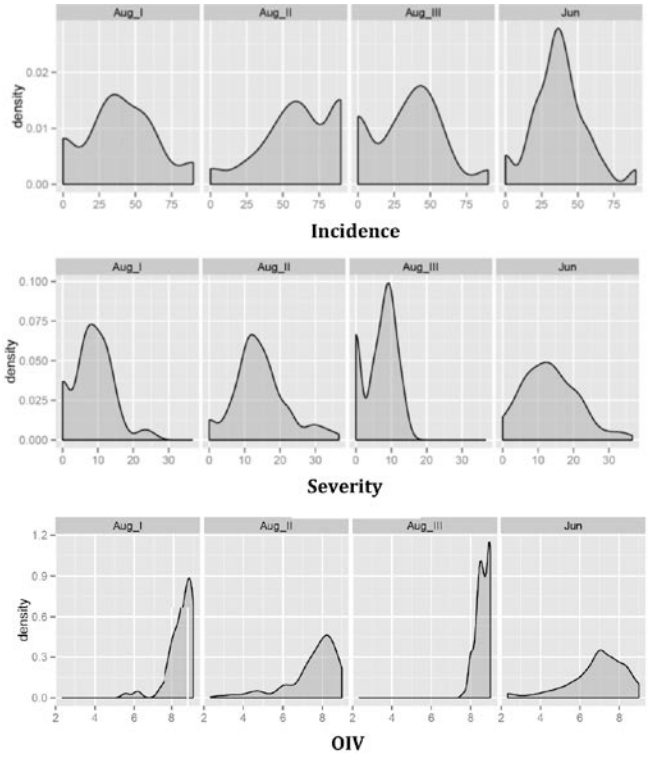

Figure 1. Distribution of the three DM resistance parameters [severity, incidence, and OIV 452 (for P) or OIV452-1 (for LD) descriptors; % data arcsin-transformed] evaluated on potted plants (P) in June (Jun) and leaf disks (LD) in August (Aug_I, Aug_II, Aug_III) of the M×T segregating population upon *P. viticola* inoculation.

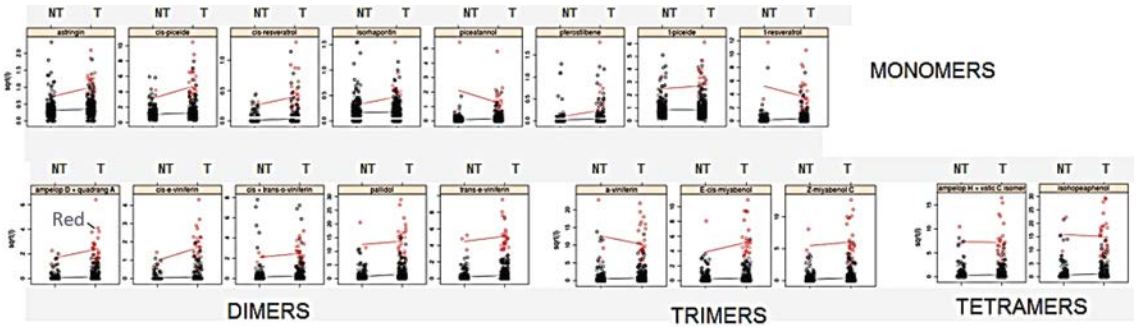

Figure 2. Distribution of 18 detectable leaf stilbenoids. After induction, significant increases (*t*-test with $p \leq 0.05$) in several stilbenoids upon *P. viticola* inoculation (T) vs. mock treatment (NT) were found (concentration data ln-transformed). Resistant F_1 individuals are depicted in red.

QTL mapping and candidate gene selection

The association between genotypic and phenotypic data allowed us to: i) identify a major QTL on LG 18 for all DM-resistance parameters, which explained up to 23% of phenotypic variance; although this region corresponds to the *Rpv3* locus previously discovered in other resistant genotypes (Di Gaspero et al., 2012), the present study led to the characterization and definition of the *Rpv3-3* haplotype as associated with DM resistance in 'Merzling'; ii) define a number of novel QTLs on 12 different LGs associated with 30 of the 64 phenolics-related parameters. In most of cases, except for a QTL on LG 12 specifically associated with gallic acid, the identified regions showed pleiotropic effects on several parameters and explained different percentages of variance, whereas, in some cases, more than one region was associated with a certain parameter.

No QTL associated with phenolics-related parameters fell into the major DM resistance QTL.

The number of genes within the identified QTL regions varied from a minimum of five (LG 18) to a maximum of 984 (LG 16). Because of the large number of gene predictions underlying the QTL regions, we adopted four criteria to identify CGs putatively associated with the traits under investigation. Candidate functions of particular interest were signaling, regulation of transcription, response to abiotic and biotic stimuli, secondary metabolism and transport. Focusing on these categories, a refined list of 88 selected CGs was generated. Of these, a few were previously associated with the DM response and the regulation of polyphenol synthesis, while the majority were identified in the present study.

Besides numerous disease-related (NBS-LRR) genes, within the major QTL on LG 18 associated with DM-resistance parameters, we found four laccases (VIT_218s0117 g00590, VIT_218s0117 g00600, VIT_218s0117 g00610, VIT_218s0117 g00625) that may be part of the defense response.

Concerning the polyphenol content trait, we focused on CGs underlying QTLs associated with polyphenols induced by infection as well as presenting a different trend between *Rpv3-3*$^{+}$ and *Rpv3-3*$^{-}$ genotypes, characterized by a different level of DM resistance. Within the category of signaling, many selected genes belong to the kinase protein family, as well as to ethylene, abscisic acid (ABA) and jasmonic acid (JA) signaling pathways. A cluster of stilbene synthase genes, mostly not specifically related to the DM response previously, was found in the QTL intervals associated with *cis*-piceid and pterostilbene. In addition, six peroxidase genes (herein named *VviPrxIII08a*, *VviPrxIII08b*, *VviPrxIII15a*, *VviPrxIII21a*, *VviPrxIII23a*, and *VviPrxIII34a*) underlay monomeric and oligomeric stilbenoid-related QTL regions and two laccase genes (*VIT_218s0001 g02400* and *VIT_218s0001 g02410*) were associated with ε-viniferin. Looking at regulators, seven *MYB* genes not yet associated with polyphenol regulation were identified: *VIT_216s0013 g01560* (*VvMYB193* in Wong et al., 2016) and *VIT_216s0013 g01570* (*VvMYB194* in Wong et al., 2016) in the region controlling both 2,6-dihydrobenzoic acid and astringin content, *VIT_204s0044 g01380* in the region

controlling astringin content, *VIT_217s0000 g02710* and *VIT_217s0000 g02730*, associated with ampelopsin D + quadrangularin A, the sum of dimeric stilbenoids and rutin content, and finally *VIT_214s0060 g02640*, related to the sum of polymeric stilbenoids. Finally, a WRKY factor (precisely VvWRKY28 in Wang et al., 2014) was associated with *cis*-resveratrol content, extending the list of WRKY factors recently identified as involved in the regulation of stilbenoid metabolism (Malacarne et al., 2018; Vannozzi et al., 2018).

Validation of the results obtained at the metabolic level came from a transcriptional investigation in a set of 12 F_1 individuals of the progeny, which highlighted a significant association between some monomeric and dimeric stilbenoids and the transcript level of three newly identified peroxidases beside known stilbene synthases (data not shown).

ACKNOWLEDGMENTS

The authors would like to thank Daniele Brazzale and Susanna Micheli for their support during inoculations and sample preparation for biochemical analysis, Rohn Wehrens for suggestions regarding the statistical analyses of phenotypic data, and Luca Cappellin for R software assistance.

Literature cited

Blasi, P., Blanc, S., Wiedemann-Merdinoglu, S., Prado, E., Rühl, E.H., Mestre, P., and Merdinoglu, D. (2011). Construction of a reference linkage map of *Vitis amurensis* and genetic mapping of *Rpv8*, a locus conferring resistance to grapevine downy mildew. Theor. Appl. Genet. *123* (*1*), 43–53 https://doi.org/10.1007/s00122-011-1565-0. PubMed

Di Gaspero, G., Copetti, D., Coleman, C., Castellarin, S.D., Eibach, R., Kozma, P., Lacombe, T., Gambetta, G., Zvyagin, A., Cindrić, P., et al. (2012). Selective sweep at the *Rpv3* locus during grapevine breeding for downy mildew resistance. Theor. Appl. Genet. *124* (*2*), 277–286 https://doi.org/10.1007/s00122-011-1703-8. PubMed

Divilov, K., Barba, P., Cadle-Davidson, L., and Reisch, B.I. (2018). Single and multiple phenotype QTL analyses of downy mildew resistance in interspecific grapevines. Theor. Appl. Genet. *131* (*5*), 1133–1143 https://doi.org/10.1007/s00122-018-3065-y. PubMed

Huang, Y.F., Doligez, A., Fournier-Level, A., Le Cunff, L., Bertrand, Y., Canaguier, A., Morel, C., Miralles, V., Veran, F., Souquet, J.M., et al. (2012). Dissecting genetic architecture of grape proanthocyanidin composition through quantitative trait locus mapping. BMC Plant Biol. *12* (*1*), 30 https://doi.org/10.1186/1471-2229-12-30. PubMed

Malacarne, G., Vrhovsek, U., Zulini, L., Cestaro, A., Stefanini, M., Mattivi, F., Delledonne, M., Velasco, R., and Moser, C. (2011). Resistance to *Plasmopara viticola* in a grapevine segregating population is associated with stilbenoid accumulation and with specific host transcriptional responses. BMC Plant Biol. *11* (*1*), 114 https://doi.org/10.1186/1471-2229-11-114. PubMed

Malacarne, G., Costantini, L., Coller, E., Battilana, J., Velasco, R., Vrhovsek, U., Grando, M.S., and Moser, C. (2015). Regulation of flavonol content and composition in (Syrah×Pinot Noir) mature grapes: integration of transcriptional profiling and metabolic quantitative trait locus analyses. J. Exp. Bot. *66* (*15*), 4441–4453 https://doi.org/10.1093/jxb/erv243. PubMed

Malacarne, G., Pilati, S., Valentini, S., Asnicar, F., Moretto, M., Sonego, P., Masera, L., Cavecchia, V., Blanzieri, E., and Moser, C. (2018). Discovering causal relationships in grapevine expression data to expand gene networks. A case study: four networks related to climate change. Front. Plant Sci. *9*, 1385 https://doi.org/10.3389/fpls.2018.01385. PubMed

Marguerit, E., Boury, C., Manicki, A., Donnart, M., Butterlin, G., Némorin, A., Wiedemann-Merdinoglu, S., Merdinoglu, D., Ollat, N., and Decroocq, S. (2009). Genetic dissection of sex determinism, inflorescence morphology and downy mildew resistance in grapevine. Theor. Appl. Genet. *118* (*7*), 1261–1278 https://doi.org/10.1007/s00122-009-0979-4. PubMed

Merdinoglu, D., Wiedeman-Merdinoglu, S., Coste, P., Dumas, V., Haetty, S., Butterlin, G., and Greif, C. (2003). Genetic analysis of downy mildew resistance derived from *Muscadinia rotundifolia.* Acta Hortic. *603*, 451–456 https://doi.org/10.17660/ActaHortic.2003.603.57.

Ochssner, I., Hausmann, L., and Töpfer, R. (2016). Rpv14, a new genetic source for *Plasmopara viticola* resistance conferred by *Vitis cinerea.* Vitis *55*, 79–81.

OIV (2009). Descriptor List for Grape Varieties and *Vitis* Species, 2nd edn (Paris, France: Office International de la Vigne et du Vin).

R Core Team. (2018). R: a Language and Environment for Statistical Computing (Vienna, Austria: R Foundation

for Statistical Computing).

Salmaso, M., Malacarne, G., Troggio, M., Faes, G., Stefanini, M., Grando, M.S., and Velasco, R. (2008). A grapevine (*Vitis vinifera* L.) genetic map integrating the position of 139 expressed genes. Theor. Appl. Genet. *116* (*8*), 1129–1143 https://doi.org/10.1007/s00122-008-0741-3. PubMed

Schwander, F., Eibach, R., Fechter, I., Hausmann, L., Zyprian, E., and Töpfer, R. (2012). *Rpv10*: a new locus from the Asian *Vitis* gene pool for pyramiding downy mildew resistance loci in grapevine. Theor. Appl. Genet. *124* (*1*), 163–176 https://doi.org/10.1007/s00122-011-1695-4. PubMed

Van Ooijen, J.W. (2006). JoinMap 4, Software for the Calculation of Genetic Linkage Maps in Experimental Populations (Wageningen, The Netherlands: Kyazma BV).

Van Ooijen, J.W. (2009). MapQTL 6, Software for the Mapping of Quantitative Trait Loci in Experimental Populations of Diploid Species (Wageningen, The Netherlands: Kyazma BV).

Vannozzi, A., Wong, D.C.J., Höll, J., Hmmam, I., Matus, J.T., Bogs, J., Ziegler, T., Dry, I., Barcaccia, G., and Lucchin, M. (2018). Combinatorial eegulation of stilbene synthase genes by WRKY and MYB transcription factors in grapevine (*Vitis vinifera* L.). Plant Cell Physiol. *59* (*5*), 1043–1059 https://doi.org/10.1093/pcp/pcy045. PubMed

Vezzulli, S., Vecchione, A., Stefanini, M., and Zulini, L. (2018). Downy mildew resistance evaluation in 28 grapevine hybrids promising for breeding programs in Trentino region (Italy). Eur. J. Plant Pathol. *150* (*2*), 485–495 https://doi.org/10.1007/s10658-017-1298-2.

VIVC. (2018). Vitis International Variety Catalogue. http://www.vivc.de/index.php.

Vrhovsek, U., Masuero, D., Gasperotti, M., Franceschi, P., Caputi, L., Viola, R., and Mattivi, F. (2012). A versatile targeted metabolomics method for the rapid quantification of multiple classes of phenolics in fruits and beverages. J. Agric. Food Chem. *60* (*36*), 8831–8840 https://doi.org/10.1021/jf2051569. PubMed

Wang, M., Vannozzi, A., Wang, G., Liang, Y.-H., Tornielli, G.B., Zenoni, S., Cavallini, E., Pezzotti, M., and Cheng, Z.M. (2014). Genome and transcriptome analysis of the grapevine (*Vitis vinifera* L.) WRKY gene family. Hortic. Res. *1* (*1*), 14016 https://doi.org/10.1038/hortres.2014.16. PubMed

Wickham, H. (2016). ggplot2: Elegant Graphics for Data Analysis (New York, NY, USA: Springer-Verlag).

Wickham, H. (2017) tidyverse: Easily Install and Load the 'Tidyverse'. R package version 1.2.1. https://CRAN.R-project.org/package=tidyverse.

Wong, D.C.J., Schlechter, R., Vannozzi, A., Höll, J., Hmmam, I., Bogs, J., Tornielli, G.B., Castellarin, S.D., and Matus, J.T. (2016). A systems-oriented analysis of the grapevine R2R3-MYB transcription factor family uncovers new insights into the regulation of stilbene accumulation. DNA Res. *23* (*5*), 451–466 https://doi.org/10.1093/dnares/dsw028. PubMed