Two-omics data revealed commonalities and differences between Rpv12- and Rpv3-mediated resistance in grapevine

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Plasmopara viticola is the causal agent of grapevine downy mildew (DM). DM resistant varieties deploy effector-triggered immunity (ETI) to inhibit pathogen growth, which is activated by major resistance loci, the most common of which are Rpv3 and Rpv12. We previously showed that a quick metabolome response lies behind the ETI conferred by Rpv3 TIR-NB-LRR genes. Here we used a grape variety operating Rpv12-mediated ETI, which is conferred by an independent locus containing CC-NB-LRR genes, to investigate the defence response using GC/MS, UPLC, UHPLC and RNA-Seq analyses. Eighty-eight metabolites showed significantly different concentration and 432 genes showed differential expression between inoculated resistant leaves and controls. Most metabolite changes in sugars, fatty acids and phenols were similar in timing and direction to those observed in Rpv3-mediated ETI but some of them were stronger or more persistent. Activators, elicitors and signal transducers for the formation of reactive oxygen species were early observed in samples undergoing Rpv12-mediated ETI and were paralleled and followed by the upregulation of genes belonging to ontology categories associated with salicylic acid signalling, signal transduction, WRKY transcription factors and synthesis of PR-1, PR-2, PR-5 pathogenesis-related proteins.

Downy mildew is one of the most destructive diseases of the grapevine, causing significant limitations on grape production in the absence of chemical protection of vineyards. Downy mildew is caused by the biotrophic oomycete Plasmopara viticola (Berk. And Curt) Berl. and Toni, which is native to North America and was introduced into Europe at the end of the nineteenth century. The European bunch grape (Vitis vinifera L.) does not normally activate the immune system in response to P. viticola with a few exceptions1,2. The introgression of resistant genes from other grape species (i.e. V. rupestris, V. amurensis, V. cinerea, V. riparia and Muscadinia rotundifolia) into the crop germplasm can alleviate the dependence of viticulture on the use of fungicides3.

The pathogen can cause serious damage to any green organ of the grapevine. P. viticola deploys a specialized structure called haustorium to establish a close interaction with grapevines and to leak nutrients from viable host cells4,5. P. viticola can feed on both susceptible and resistant grapevines and complete its life cycle on both hosts. Resistant grapevines are, however, able to detect the invading pathogen and operate defence responses. The initial phases of the infection are similar on both hosts, but mycelial growth is restricted in resistant hosts and sporangia are released at lower rates than in susceptible hosts. The similarity during early phases of infection suggests the presence of post-infection mechanisms of resistance, including callose deposition, cell wall-associated

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defence processes, accumulation of reactive oxygen species and hypersensitive response (HR) associated with necrosis, and production of phytoalexins and antimicrobial compounds. These responses are paralleled by the up-regulation of genes coding for pathogenesis-related (PR) proteins. The study of the defence response in resistant grapevines can improve our understanding of the resistance mechanisms and shed light on the metabolic pathways involved. It is commonly believed that ETI requires a massive reallocation of energy and materials towards a multitude of actions against pathogen colonisation, which reduce fitness in annuals and might be energetically costly in perennial crops grown under limiting conditions.

We recently performed metabolomic analyses after pathogen inoculation in the grape variety ‘Bianca’ that carries a major resistance haplotype called Rpv3-1, leading to the identification of 53 metabolites associated with the defence response. We decided to extend this investigation using another resistant grapevine that has a genetic background highly similar to ‘Bianca’. This grapevine shares a complete haploid complement with its parent ‘Bianca’ but not the resistance haplotype Rpv3-1 at the Rpv3 locus on chromosome 18 and it carries instead a different resistance gene—Rpv12—that is located on chromosome 14. We first identified a suitable genotype for this kind of experiment in an accession obtained in 2000 by P. Kozma and S. Hoffmann in Hungary from a cross between ‘Bianca’ and the breeding line SK77-4/5 and held in a germplasm repository in South Tyrol (Italy). The resistance haplotype at the Rpv12 locus was initially introgressed from the Asian species Vitis amurensis and confers higher levels of downy mildew resistance than Rpv3-1 when the resistance phenotype is quantitatively scored using the OIV452 descriptor, but both genes trigger a similar ETI-dependent response. The donor parent of the Rpv12 resistance haplotype, SK77-4/5, was bred at the University of Novi Sad, Serbia, by crossing V. vinifera ‘Traminer’ and the resistant variety ‘Kunbarat’, which descends from the interspecific hybridization of V. amurensis and V. vinifera. In our previous study in ‘Bianca’, we focused on Rpv3-mediated metabolome changes upon pathogen inoculation with the aim to identify biomarkers of resistance. Recently, Eisenmann and coworkers performed a transcriptomic analysis in ‘Regent’, a resistant variety that operates the same Rpv3-mediated ETI response as does ‘Bianca’, showing that Rpv3-mediated changes in gene expression occur early after pathogen inoculation.

Technological developments in genomics, transcriptomics, proteomics and metabolomics have provided powerful tools for systems biology studies. Metabolomics is widely used for monitoring changes in response to the developmental program, to the environment, and to biotic stresses. In grapevine, metabolomics has been used to investigate several aspects of berry development and berry ripening or to study the effects of abiotic stresses or to study the effects of abiotic stresses or to study the effects of abiotic stresses or to study the effects of abiotic stresses or to study the effects of abiotic stresses or to study the effects of abiotic stresses or to study the effects of abiotic stresses or to study the effects of abiotic stresses or to study the effects of abiotic stresses or to study the effects of abiotic stresses or to study the effects of abiotic stresses or to study the effects of abiotic stresses or to study the effects of abiotic stresses or to study the effects of abiotic stresses or to study the effects of abiotic stresses or to study the effects of abiotic stresses or to study the effects of abiotic stresses or to study the effects of abiotic stresses or to study the effects of abiotic stresses or to study the effects of abiotic stresses or to study the effects of abiotic stresses or to study the effects of abiotic stresses. More recent studies using grape leaves that had been inoculated with P. viticola have used metabolomic analyses to monitor specific classes of compounds or a limited number of molecules. In our previous work, we extended the number of compounds that are identified and quantified in grape leaves using a single analytical run. Transcriptomics refers to the global analysis of all transcribed genes in a certain organ. Transcriptomic analyses based on RNA-Seq (RNA-Seq) provide a comprehensive survey of gene expression changes occurring during the response to pathogen infections. Transcriptomic analyses have been performed on grape leaves undergoing defence responses to P. viticola in native American species and in V. vinifera introgression lines carrying Rpv3-1, Rpv3-3, or Rpv10 resistance genes. Using a single-omics approach it is possible to clarify only one aspect of cell metabolism. Multi-omics analyses offer a unifying view of the biological processes and have been applied to fruit crop species for inferring relationships between genes, transcripts, metabolites and phenotypes. Recent work has been done in grapevine by applying a multi-omics integrated approach to study drought stress, berry developmental changes, light exclusion, basal immunity and responses to Erysiphe necator infection. Here we present two integrated omics data of Rpv12-mediated ETI responses to P. viticola in grapevine leaves and compare the results with our previous data and other literature reports in which the same two-omics were used separately for studying Rpv3-mediated responses.

Results

Impact of the defence response on leaf metabolome. We identified and quantified 175 metabolites in inoculated and control leaves. A complete list of these compounds and their concentration is reported in Supplementary Table S1, sorted into four classes of primary metabolites, lipids, phenols, and volatile compounds. Figure 1 illustrates a lower-dimensional picture of the distribution of samples undergoing Rpv12-mediated ETI responses and controls during the course of the experiment using a Principal Component Analysis (PCA). The first two components explained 62.8, 58.1, 52.2, and 70.0% of the observed variation for primary metabolites, lipids, phenols, and volatile compounds, respectively. The first component (PC1) revealed a separation between inoculated samples and controls at 12 and 96 h post infection (hpi) for primary metabolites and volatile compounds (Fig. 1a,d), at 24 and 48 hpi for lipids (Fig. 1b), and at 48 and 96 hpi for phenols (Fig. 1d). The second component (PC2) separated the samples according to the stage of incubation.

With regard to primary metabolism, we observed two distinct temporal patterns of activation. A rapid and transient increase in the concentration of some sugars and most of organic and amino acids was detected at 12 hpi (Fig. 2). A delayed and more sustained increase of almost all sugars, organic acids and amino acids occurred from 48 to 96 hpi (Fig. 2). We observed the least differences in primary metabolites between inoculated samples and controls at the stage of 24 hpi with the notable exception of benzoic acid that was greatly reduced in samples undergoing Rpv12-mediated defence (Fig. 2d). Benzoic acid is the precursor of salicylic acid (SA) in the phenylalanine ammonia-lyase (PAL) pathway. We therefore assume that the benzoic acid pool is depleted at 24 hpi by a rapid conversion of this substrate to SA. The concentration of fatty acids also increased in samples undergoing Rpv12-mediated defence (Fig. 3a). Linolenic acid (18:3) was among the fatty acids with elevated concentration after pathogen inoculation.
oxygen species (ROS)—and is important for the modulation of the strength of the HR41 if not responsible itself for the onset of the oxidative burst in programmed cell death (PCD)42. In a context of an elevated lipid metabolism (Fig. 3a), we observed a marked decrease in palmitoleic acic (16:1), linoleic acid (18:2), linolenic acid (18:3), and especially in oleic and vaccenic acids (18:1) at the stage of 24 hpi. It is noteworthy that the concentration of the same five fatty acids showed the most remarkable decreases in samples undergoing

It has been previously shown that Rpv3-mediated defence is characterised by the two following early events: PCD occurs in the mesophyll cells underneath the infected stomata and non-toxic stilbenes, mainly trans-resveratrol, accumulate in leaves undergoing PCD. Elevated trans-resveratrol synthesis and PCD are operated between 12 and 24 hpi and their outcomes become effective, visible, and measurable between 24 and 48 hpi12,20,23. In Rpv12-mediated ETI, the rapid accumulation of this biomarker from 12 to 96 hpi suggests a more prompt and prolonged activation of the defence response compared to Rpv3-mediated ETI.

In order to identify the metabolites that better explain the differences between inoculated samples and controls, we selected 88 metabolites that showed an absolute value of the t statistic greater than 3 (|t|> 3) in one time point at least (Supplementary Fig. S2a, Supplementary Table S2). We summarized this information in the network represented in Supplementary Fig. S2b. We identified the largest number of nodes in the network at 48 hpi, indicating that the effects of metabolic reprogramming in samples undergoing Rpv12-mediated defence were maximal between 24 and 48 hpi and these changes involved mainly secondary metabolites.

Key metabolite changes in secondary metabolism. It has been previously shown that Rpv3-mediated defence is characterised by the two following early events: PCD occurs in the mesophyll cells underneath the infected stomata and non-toxic stilbenes, mainly trans-resveratrol, accumulate in leaves undergoing PCD. Elevated trans-resveratrol synthesis and PCD are operated between 12 and 24 hpi and their outcomes become effective, visible, and measurable between 24 and 48 hpi12,20,23. The conversion of non–toxic trans-resveratrol into fungi–toxic derivatives, mainly ε-viniferin, reached a peak between 48 and 96 hpi20,23. In the current experiment, the amount of trans-resveratrol was consistently two to threefold higher from 12 to 48 hpi in leaves undergoing
Figure 2. Major classes of primary metabolites in leaves undergoing *Rpv12*-mediated defence (inoculated) and controls. Cumulative concentration of (a) sugars, (c) organic and (e) amino acids. (b,d,f) Report the log2 ratio of the concentration of each compound in inoculated leaves versus controls.

Figure 3. Differences in fatty acids between leaves undergoing (a) *Rpv12*- or (b) *Rpv3*-mediated defence. On the y-axis the log2 ratio of the concentration of each compound in inoculated leaves versus controls is shown. Graph in (b) was plotted using raw data obtained from our previous paper20.

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Rpv12-mediated resistance than in controls (Fig. 5a), whereas this increase occurred more transiently from 12 to 24 hpi in our previous experiment with Rpv3-mediated resistance (Fig. 5c). As a result of this, the concentration of trans-resveratrol stayed within a higher range of variation (between 1.8 and 3.1 mg kg\(^{-1}\) of fresh weight (FW)) during the first 48 hpi in leaves undergoing Rpv12-mediated resistance, whereas the concentration remained within a lower range (between 0.7 and 1.7 mg kg\(^{-1}\) of FW) in Rpv3-mediated resistance\(^{20}\). This strong increase of trans-resveratrol in Rpv12-mediated resistance compared to controls was not paralleled or followed by a commensurate increase in ε-viniferin, which originates from oxidative dimerization of trans-resveratrol.

The concentration of ε-viniferin was higher in inoculated samples undergoing Rpv12-mediated resistance than in controls only at 12 hpi (Fig. 5b), whereas it markedly increased at 48 and 96 hpi in Rpv3-mediated resistance (Fig. 5d).

Impact of the defence response on the leaf transcriptome and global changes in metabolic pathways. Figure 6 illustrates a lower-dimensional picture of the distribution of samples undergoing Rpv12-mediated defence response and controls during the course of the experiment based on a PCA using gene expression values for all transcribed genes. Replicates of samples undergoing Rpv12-mediated defence response at 24 hpi grouped separately from replicated controls along the first component (PC1, Fig. 6a). Replicates of samples undergoing Rpv12-mediated defence responses grouped separately from replicated controls at 48 and 96 hpi.
along the third component (PC3, Fig. 6b). Figure 7 illustrates the distributions of FPKM log2 ratios between samples undergoing *Rpv12*-mediated ETI and controls at each stage of incubation for 12 groups of genes sorted by gene ontology (GO) category and/or metabolic pathway. Genes that are involved in sugar metabolism and generation of energy showed small global shifts in expression between samples undergoing *Rpv12*-mediated ETI and controls. The distributions of FPKM log2 ratios for sugar metabolism genes (Fig. 7a) were shifted towards negative median values from 12 to 48 hpi and were significantly shifted towards higher values and with a positive median value only at 96 hpi, suggesting that sugar metabolism pathways were slightly modulated at early stages in samples undergoing *Rpv12*-mediated ETI response. However, categories including genes that encode sugar transporters (Fig. 7b) and are involved in fatty acid metabolism (Fig. 7f) showed the most remarkable global shift in FPKM log2 ratios towards higher and positive values at 12 and 24 hpi. This is consistent with the notion that the activation of sugar signaling and fatty acid modulators of signal transduction pathways relies on mobilization and deployment of existing resources at the initial stages of the ETI response. These metabolic activities were sustained by the activation of pathways involved in generation of energy as we observed slight global shifts of distributions of FPKM log2 ratios for genes assigned to energy-related gene categories towards positive median values at 12, 24, and 96 hpi (Fig. 7c). Although the ETI response seems to require substantial amounts of carbon and energy, the global expression of genes involved in photosynthesis (Fig. 7d) suggested that these resources were not contributed by an activation of the photosynthetic pathway that was on the contrary strongly reduced at 48 and 96 hpi. We observed in samples undergoing *Rpv12*-mediated ETI two distinct temporal patterns of elevated expression of the gene family encoding WRKY transcription factors (Fig. 7i). A rapid and transient increase was detected at 12 hpi and was followed by a more sustained increase from 48 to 96 hpi. WRKY transcription factors are known to activate defence response in a number of ways through the up-regulation of genes involved in the phenylpropanoid pathway and in SA synthesis and signalling. Indeed, we found that structural genes of the phenylpropanoid and downstream pathways—including those required for stilbene synthesis—showed global shifts in the distributions of FPKM log2 ratios towards positive median values at 12 and 96 hpi (Fig. 7g). This observation recalls the notion that compounds in this class activate early signalling pathways and, at later stages of the incompatible interaction, sustain the production of stilbenes. We also observed statistically significant shifts towards higher FPKM log2 ratios with positive median values in the distribution for genes involved in SA synthesis and signalling at 48 and 96 hpi (Fig. 7j), concomitant with similar increases in the expression of genes involved in calcium and mitogen activated protein (MAP) kinase signalling (Fig. 7k). We finally used the set of genes assigned to the GO categories of cell cycle and cell homeostasis that are not expected to be recruited in defence responses as a negative control for this analysis. We indeed observed that these GO categories showed no statistically significant difference in their global distributions of FPKM log2 ratios between stages and their median log2 ratios pointed to zero at each stage of incubation (Fig. 7l).

**Statistically significant and major differences in individual gene expression.** We then defined a set of differentially expressed genes (DEGs) between inoculated samples and controls by selecting genes with an absolute value of log2 Fold Change (FC) in expression greater than 1 and adjusted *p* value *p* < 0.05. We found 431 DEGs including alternative transcripts with differential expression for 35 genes (Supplementary Table S3). We validated by quantitative PCR (qPCR) a sample of DEGs representative of different GOs and found consistency both in the log2 ratios (Supplementary Fig. S3) and in the statistical significance of the difference (Sup-
between samples undergoing Rpv12-mediated ETI and controls. Only two DEGs were identified at 12 hpi, which were both up-regulated in samples undergoing Rpv12-mediated defence responses. These two genes are VIT_207s0129g00660 encoding an Indole-3-acetic acid (IAA)-amido synthetase (IAA) and VIT_209s0002g06890 encoding a blue copper protein. IAA-amido synthetases, also known as GH3 proteins, dampen free-IAA levels by catalysing the conjugation of IAA to amino acids. GH3-overexpressing plants in Arabidopsis and rice showed enhanced resistance to Pseudomonas syringae<sup>45</sup> and Magnaporthe oryzae<sup>46</sup>, respectively, and it is generally assumed that suppression of IAA signalling is an integral part of SA-mediated resistance against biotrophic pathogens<sup>47,48</sup>. The second up-regulated gene (VIT_209s0002g06890) showed elevated expression levels also at 24 and 48 hpi. We found 70 DEGs at 24 hpi (17 up-regulated; 53 down-regulated in samples undergoing Rpv12-mediated defence responses); 50 DEGs at 48 hpi (41 up-regulated; 9 down-regulated in samples undergoing Rpv12-mediated defence responses) and 340 genes at 96 hpi (326 up-regulated; 14 down-regulated in samples undergoing Rpv12-mediated defence responses). The expression of a fraction of genes was statistically different between samples undergoing Rpv12-mediated defence responses and controls at more than one stage of incubation as reported in the Venn diagram of Supplementary Fig. S4. We focused our attention on the top 100 most significantly enriched GO categories for biological processes represented in the list of DEGs and the 20 GO categories with lowest p values are shown in Supplementary Table S5. At 48 and

Figure 7. Global differences of gene expression in leaves undergoing Rpv12-mediated defence (inoculated) and controls. Genes were grouped by metabolic pathways (a–l). Box–plot distributions of FPKM log<sub>2</sub> ratios at four stages of incubation. Each letter identifies distributions that are not significantly different using a Wilcoxon–Mann–Whitney test (P < 0.05). Alphabetical order indicates increasing median values. The line pointing to the median value in each box is plotted as a solid line for positive median values and as a dotted line for negative median values.
At 96 hpi, we found up-regulation of DEGs belonging to the “plant hypersensitive response” (GO:0009626). We also found up-regulation for DEGs belonging to GO categories such as the “salicylic acid biosynthetic process” (GO:0009697), “systemic acquired resistance, salicylic acid mediated signalling pathway” (GO:0009862) and “response to salicylic acid stimulus” (GO:0009751). It was worth noting that at the same stages of incubation we found a significant up-regulation of the gene VIT_208s0058g01390 encoding a WRKY70 transcription factor that acts in Arabidopsis as an activator of SA-induced PR genes and is required for RPP4-mediated disease resistance against Hyaloperonospora parasitica49. It is known that resistance genes activate ETI responses through the SA-dependent signalling pathway and through signalling cascades that amplify the spectra of defence responses. Indeed, we found up-regulation of DEGs belonging to the “MAP kinase cascade” category (GO:0000165). For instance, the gene VIT_209s0002g04560 encoding a calmodulin-binding protein was significantly up-regulated at 48 and 96 hpi in samples undergoing Rpv12-mediated defence responses. Calmodulin-binding proteins play crucial roles in cellular signalling cascades through the regulation of numerous target proteins50. We also observed up-regulation of the gene VIT_203s0088g00910 encoding a class 1 pathogenesis related protein (PR-1) at 24, 48 and 96 hpi and of the gene VIT_202s0025g04330 encoding a thaumatin-like class 5 protein (TLP or PR-5) at 48 and 96 hpi in samples undergoing Rpv12-mediated defence responses (Supplementary Table S4). At 24 and 48 hpi we also found up-regulation of DEGs VIT_208s0007g06040 and VIT_208s0007g06060 encoding beta-glucanases that operate cell wall lysis of invading pathogens as a part of the inducible defence responses. DEGs with predicted functions related to amino acid, carbohydrate and lipid metabolism were up-regulated at 96 hpi together with genes belonging to secondary metabolism pathways within the GOs “coumarin biosynthetic process” (GO:0009805), and “stilbene biosynthetic process” (GO:0009811). At 96 dpi, we also found significant down-regulation of individual genes involved in photosynthesis and photosynthetic electron transport chain processes, such as VIT_210s0003g02900, VIT_217s0000g06350, VIT_216s0003g02890 encoding different chlorophyll a-b binding proteins.

**MCIA of metabolites and transcripts.** We used Multiple Co-Inertia Analysis (MCIA), a method developed by Meng et al.31, to identify co-relationships between the two-omics datasets using a multivariate dimension reduction approach. MCIA rescales the values from heterogeneous datasets using a covariance criterion and projects simultaneously multiple datasets onto the same plane. The results of MCIA analysis are reported in Fig. 8. Individual transcripts and metabolites are represented by grey filled squares and black filled circles, respectively, regardless of the sample type, of the stage of incubation and of the replicate. The colored dots, which are differentiated by the stage of incubation, recapitulate the global distribution of each omics dataset on the same bidimensional space, sorted by sample type and replicate. Large colored triangles and circles recapitulate the integration of the two-omics datasets, sorted by sample type, stage of incubation and replicate. The length of the connectors between large symbols and the corresponding small colored dots are inversely correlated with the extent to which the two-omics datasets are coherent in the separation by sample type, stage of incubation...
and replicate on the bidimensional space. The MCIA plot showed that the strongest separation between samples undergoing Rpv12-mediated defence responses and controls occurred at 96 hpi.

**Discussion**

In grapevine leaf discs that are operating Rpv12-mediated resistance against the infection of *P. viticola*, the host tissues are characterised by wider and faster metabolome changes than those observed in a similar experiment performed on leaf discs that were operating Rpv3-mediated resistance. Using the same analytical and statistical methods and the same cut-off thresholds, we identified a higher number of metabolites (88 versus 53) that were significantly modulated over a 96 h period after the inoculation with the pathogen. We also found that substantial metabolite changes occurred earlier in Rpv12-mediated resistance compared to Rpv3-mediated resistance with the highest number of modulated metabolites at 48 hpi instead of 96 hpi. Although we paid particular attention to the choice of an appropriate Rpv12-carrying genotype in order to minimise the confounding effects of the genetic background, we cannot exclude that part of the faster and stronger defence responses of the Rpv12-carrier compared to the Rpv3-carrier was due to more effective signal transduction components downstream of the R-gene in the genotype under study. We indeed observed substantial variation in field resistance against natural infections of *P. viticola* among dozens of grape varieties that all carry the same Rpv3-I resistance haplotype in an ample variation of genetic backgrounds. While for choosing the Rpv3-carrier we had background information available to select an average variety, such as ‘Bianca’, as a representative of a known distribution, similar data are still not available for a substantial number of Rpv12-carriers, due to a more recent origin of this lineage. While Rpv3-carrying varieties had been introduced in cultivation since the late 1800’s and since then used for further breeding, it was only in 1960 that Koleda and Tamássy backcrossed *V. amurensis* × *V. vinifera* hybrids to generate the first two resistant varieties (‘Kunleany’ and ‘Kunbarat’) carrying a new resistance that later has become known as Rpv12-dependent. It took another 15 years after Koleda and Tamássy, before Ćindrič and Korać had the intuition that those two varieties could become excellent progenitors for a new lineage of resistant varieties, performing in 1977 the backcross of ‘Kunbarat’ that has led to origin of the individual SK77-4/5, a parent of the individual of this study. It is therefore possible that we used by chance, among the few available, an Rpv12-carrier with a particularly favourable genetic background, although several reports converge on a consensus that, based on OIV452 scores, Rpv12-dependent resistance is stronger across varieties than Rpv3-dependent resistance. Bove and Rossi showed that three varieties operating Rpv12-dependent defence clustered together and separately from varieties carrying other Rpv genes for five defence components (i.e. proportion of inoculated sites that develop disease symptoms, length of the latent period, number of waves of sporangial release during the infectious period upon extended incubation of sporulating lesions, sporangial density per lesion, infectivity of the released sporangia), suggesting that the outcome of the defence response is still more dependent on the type of R-gene than on the genetic background of the variety. Despite the inherent limitations of this kind of comparative experiments, we observed that changes leading to a common metabolic reprogramming necessary for triggering and operating cell death at the infection sites and for SA-dependent synthesis of PR-proteins have occurred in Rpv12-mediated resistance with similar timing and in the same direction as observed for Rpv3-mediated resistance.

While the causal factor that triggers Rpv3-mediated HR has been identified in the gene products of two nearby TIR-NB-LRRs, the causal gene that would explain the observed Mendelian inheritance of Rpv12-mediated resistance has not been isolated yet. The sequence of the reference grapevine genome that has been assembled from a DM sensitive genotype predicts the presence of allelic forms of CC-NB-LRR genes in the Rpv12 locus. Although the Rpv12-resistance haplotype has yet to be sequenced, the Rpv12-mediated response analysed in this paper shows the hallmarks of a ROS- and SA-dependent HR triggered by a NB-LRR gene product. Here, we provided evidence that gene expression profiling and changes in transcription levels of gene categories grouped by metabolic pathways lend support to the observed metabolite changes over a period of 96 hpi and highlight striking similarities with the canonical model of ETI-mediated defence responses to biotrophic pathogens in plants. Indeed, cumulative evidence from metabolomic and transcriptomic data suggests that a Rpv12-mediated resistance relies on a rapid activation of a broad set of inducible responses that take place within 12 hpi. At 12 hpi, we detected elevated expression of gene categories encoding sugar transporters, which suggest a mobilisation of existing carbon resources, and an increase of sugar concentration. Sugar sensing and sugar-induced signalling have been shown to activate pathways leading to the synthesis of PR-proteins, to elicit PCD and to induce a metabolic swift from source to sink through a negative feedback loop. At 12 hpi, samples undergoing Rpv12-mediated ETI also showed elevated concentration of linolenic acid that is reported to directly activate NADPH-oxidase, an enzyme required for oxidative burst. An increase in linolenic acid has also been observed at the early stages of the Rpv3-mediated defence response in ‘Regent’. Elevated levels of *trans*-resveratrol were observed at 12 hpi in both Rpv12- and Rpv3-mediated defence responses. In contrast with metabolite and transcript analyses of Rpv3-mediated and of Rpv10-mediated defence, we did not find statistically significant differences between samples undergoing Rpv12-mediated defence response and controls in the expression of the gene VIT_21200028g01840, which encodes a peroxidase possibly involved in the dimerization of *trans*-resveratrol into the fungi-toxic *viniferin*. Nor did we find evidence for a significant increase of *viniferin* concomitant with or subsequemt to the increase of *trans*-resveratrol in samples undergoing Rpv12-mediated defence response. Although the two roles are not mutually exclusive, these lines of evidence in Rpv12-mediated resistance point to a primary role of *trans*-resveratrol as a signalling molecule in ROS formation and initiation of programmed cell death, as proposed by, rather than as a precursor of toxic stilbenes, as it occurs in Rpv3- and in Rpv10-mediated defence. An augmented and prolonged accumulation of resveratrol, without a substantial conversion into *viniferin*, has also been observed in otherwise sensitive grapevines after they were administered a specific
sulphated derivative of laminarin that primed H$_2$O$_2$ production and suppressed the development of $P$. viticola in inoculated leaf discs$^{45}$. This ample set of early changes that had already occurred by 12 hpi in samples undergoing Rpv12-mediated defence suggests that the metabolism is timely reconfigured shortly after pathogen perception and the entire defence machinery comes immediately into operation as part of a crisis management program. However, the full deployment of the activated defence weaponry remains on standby until a point of no return that is surpassed when ROS and SA synergistically potentiate the response and drive cells into an auto-toxic and energetically costly process. At 24 hpi, we observed strong reduction in 18:1 fatty acids that suggests initiation of ROS production, as it occurs in the Rpv3-mediated defence response with a timing that coincides in that case with the execution of PCD$^{23}$. In Arabidopsis, it is reported that lower levels of 18:1 fatty acids directly stabilise the NITRIC OXIDE ASSOCIATED1 enzyme, increasing nitric oxide concentration$^{65}$, a signalling gaseous radical that potentiates ROS-dependent PCD and up-regulates gene expression in the phenylpropanoid and SA pathways. It is possible that we only detected early transcriptional and metabolic signals associated with the onset of PCD at 24 hpi. Light exclusion—an experimental condition imposed for minimising the effect of light-dependent circadian fluctuations on the transcriptome at different sampling stages—might have prevented us from observing the occurrence and consequences of light-dependent oxidation, including lipid peroxidation$^{40}$, and ROS production that are known to regulate the strength of PCD-dependent HR. However, we observed a similar trend of changes in fatty acid composition in response to Rpv12-mediated defence response as that reported by$^{40}$ in Rpv3-mediated defence response in 'Regent’, which did not occur in sensitive host controls$^{40}$. These authors reported a transient and slight increase of oleic, linoleic and linolenic acids at the earliest stages of the defence response (6 hpi), followed by a rapid decrease of their concentrations at 12 hpi$^{40}$. We observed a delay in this trend, with slight increases at 12 hpi and decreases at 24 hpi, but the drop at 24 hpi was much more pronounced and consistent with what we previously observed in Rpv3-mediated defence response in ‘Bianca’$^{20}$.

We observed at 48 hpi full deployment of important components of the defence weaponry, such as SA-signalling, calcium and MAP kinase signal transduction, PR gene expression, and strong repression of photosynthesis that marked the complete source-to-sink reconfiguration of the metabolism to shuttle resources into energy-generating pathways and to prioritize the synthesis of defence-related compounds. The hypothesis of a central role of SA-signalling in eliciting some components of the Rpv12-mediated response was corroborated by the observation of a general up-regulation of the WRKY family of transcription factors. Noteworthy was the up-regulation of grapevine homologs of WRKY28 that binds to the promoter of isochorismate synthase$^{66}$, the rate-limiting enzyme in SA biosynthetic, and of WRKY70, a component of gene-for-gene resistance against the oomycete $Hyaloperonospora parasitica$ in Arabidopsis that causes induction of genes encoding PR-1, PR-2, and PR-5 proteins$^{40,66}$. The grapevine VvWRKY28 has also been proposed as a positional candidate residing in a QTL confidence interval that explained 10.8% of the phenotypic variation for cis-resveratrol content in a segregating population$^{66}$. In the same work, some stilbenoids showed a general trend of augmented concentration in individuals with higher OIV-452 scores and OIV-452 scores were higher in full-sibling individuals that carried an Rpv3-3 haplotype than in those who did not. However, no significant QTL for stilbenoid concentration was found to colocalise with the QTL for OIV-452 scores, which corresponded to the Rpv3 locus and explained 19.7% of the observed variation in $P$. viticola resistance$^{68}$. Another transcription factor, VvWRKY33, was found by$^{69}$ to be induced in ‘Regent’ during the Rpv3-mediated response from 2 to 120 hpi, with highest differences in expression levels compared to mock-inoculated controls between 4 and 10 hpi and correlation with increased expression of a pathogenesis related protein 10.1 (PR-10.1) gene, VIT$_{208s0058g00690}$ the most similar reference gene for the WRKY33 sequence isolated from ‘Regent’ (GenBank accession no. KF800706.1$^{69}$). We found elevated levels of VIT$_{208s0058g00690}$ expression at 48 and 96 hpi in leaf discs undergoing the Rpv12-mediated response compared to controls (log, Fold Change 0.70 and 0.88, respectively for the primary transcripts), although these differences remained below the threshold of statistical significance. In a similar way, we found a trend of augmented levels, yet not statistically significant, of three PR-10.1 gene copies (VIT$_{05s0077g01530}$, VIT$_{05s0077g01580}$, VIT$_{05s0077g01560}$) within a tandemly arrayed gene cluster at 48 hpi (log, Fold Change 0.19, 0.16, 0.16, respectively) and 96 hpi (log, Fold Change 0.19, 0.23, 0.23, respectively) in leaf discs undergoing the Rpv12-mediated response. In the Rpv12-mediated response, we vice versa found statistically significant induction of a thaumatin-like protein gene similar to a $V. amurensis$ ortholog that impaired mycelial growth, causing hyphal malformation, in transgenic lines of sensitive controls$^{40}$. An increasing number of yet genetically uncharacterized sources of $P$. viticola resistance have emerged in the past years from Asian species, which include $V. pseudoreticulata$ and $Vitis quinquangularis$$^{72}$. Further studies will be needed to clarify if these novel downy mildew resistances share the same type of ETI and follow the same lines of defence reactions as those observed in Rpv12-mediated resistance.

Materials and methods

Plant material and artificial inoculation. Dormant canes for the experiment were obtained from mother plants held in a germplasm repository in the South Tyrol region, Italy. Two bud cuttings ($n=45$) were rooted in potted soil and forced to sprout in isolation within a greenhouse free from sources of inoculum. A single shoot was raised from each rooted cutting until the stage of 12 expanded leaves. Water was supplied by capillary action by placing the pots, containing the rooted cuttings, onto a layer of capillary matting that was kept constantly wet. We then sorted the plants into three homogenous groups. Each group of plants ($n=15$) formed a biological replicate. At the time of the inoculation the plants were healthy, with no symptoms of any foliar disease. In parallel, shoots were raised from three cuttings of the variety ‘Pinot Noir’ in order to prepare sensitive controls. The three distal fully expanded leaves were detached from all plants of a biological replicate, bulked, and rinsed with ultrapure water. Discs were excised from each leaf lamina using a 1.1-cm diameter cork
borer and plated randomly with the abaxial surface upside on wet paper in Petri dishes, collectively representing the biological replicate. Leaf discs were acclimated at 21 °C in the dark for 12 h in order to dispel wounding-induced jasmonic acid signalling. Petri dishes from each biological replicate were divided randomly into two groups, one used for pathogen inoculation and one used for mock-inoculation, hereafter referred to as controls. Inoculated samples were sprayed with a P. viticola suspension of 10^5 sporangia ml^−1, while controls were sprayed with ultrapure water. P. viticola was originally collected as a natural mixture of sporangia from diverse V. vinifera cultivars in an unsprayed vineyard in San Michele all’Adige, Northern Italy. Sporangia were stored at −20 °C prior to use for the experiment of this study and for the experiment reported in, which was conducted two weeks earlier during the same growing season. The inoculum was propagated on plants of V. vinifera ‘Pinot Gris’ grown in potted soil in a greenhouse by spraying a 5 × 10^5 ml^−1 suspension of the frozen sporangia in distilled water. Plants were maintained at >90% relative humidity at 21 °C until sporulation. Fresh sporangia were collected from ‘Pinot Gris’ plants by soaking sporulating leaves in refrigerated (4 °C) distilled water. The suspension was diluted to the final concentration using a Burker chamber and sprayed immediately on the experimental leaf discs. All Petri dishes were incubated at 21 °C in the dark until sampling. Samples were collected at 12, 24, 48 and 96 hpi and immediately frozen in liquid nitrogen. A set of five Petri dishes was available per sampling stage, per treatment and per biological replicate. Four Petri dishes in each set collectively containing a minimum of 100 leaf discs were used for metabolomic analyses by pooling and grinding together all discs of the biological replicate. The remaining Petri dish of each set containing a minimum of 25 leaf discs was used for transcriptomic analyses by sampling two discs per time point that were pooled and ground together for RNA extraction. The remaining leaf discs were incubated until sporulation to confirm P. viticola infection on inoculated samples and to exclude P. viticola contamination prior to sample collection on mock-inoculated controls. Sporulation was not observed on inoculated Rpv12-resistant leaf discs during the course of sampling and until 120 hpi. The resistance phenotype scored a value of 9 on the OIV452 descriptor scale, due to concomitant absence of sporulation and presence of barely visible necrotic spots. Sporulation was already visible at the last stage of sampling (96 hpi) in inoculated control leaf discs of the sensitive ‘Pinot Noir’ and was confirmed after the conclusion of the experiment at 120 hpi. Sporulation was not observed on mock-inoculated control leaf discs of ‘Pinot Noir’ until 120 hpi. Gene expression analyses were performed at the genomics facility of the Fondazione Edmund Mach. Three biological replicates per treatment per time point were used for metabolite and gene expression analyses.

Metabolite analysis. Primary metabolites were determined following the methodology published by Chitarrini and coworkers. GC/MS analysis was performed by injecting 1 µL of derivatised extract into a Trace GC Ultra instrument combined with a TSQ Quantum GC mass spectrometer (Thermo Electron Corporation, MA). Compounds were separated using an RXI-5-Sil MS w/Integra-Guard column. Data acquisition was run in full scan mode from 50 to 700 m/z. Data were processed using the XCALIBUR 2.2 software.

Lipids were determined using the method developed by Della Corte et al. Chromatographic analysis was carried out using a UHPLC Dionex 3000 instrument (Thermo Fischer Scientific, DE) combined with an API 5500 triple quadrupole mass spectrometer (Applied Biosystems/MDS Sciex, CA). Compounds were separated using an RP Ascentis Express column.

Phenols were determined with slight modifications of the method developed by Vrhovsek et al. Chromatographic analysis was carried out using a Waters Acquity UPLC instrument combined with a Waters Xevo triple quadrupole mass spectrometer detector. Compounds were separated using a Waters Acquity HSS T3 column. Data processing was performed using Waters MassLynx V4.1 software.

Primary metabolites, lipids, and phenols. Compounds were identified based on their reference standard, retention time and qualifier and quantifier ion. Compounds were quantified using linear calibration curves and expressed as mg kg^−1 of FW.

Volatile compounds were extracted with slight modifications of the methods developed by Matarese et al. and Salvagnin et al. as described by Chitarrini et al. Compounds were captured from the headspace using a Supelco 2-cm DVB/CAR/PDMS 50/30 µm fibre (Supelco, PA). Chromatographic analysis was carried out using a Trace GC Ultra gas chromatograph combined with a Quant XL MS mass spectrometer (Thermo Scientific, Electron Corporation, MA). Compounds were separated using a Stabilwax-DA column (Restek Corporation, PA). Data were processed using the XCALIBUR 2.2 software. Identification of volatile compounds was carried out by injecting pure reference standards when available or comparing retention index and mass spectra using the NIST MS Search 2.0 database. Results were expressed in µg kg^−1 of FW with semi-quantification using 1-heptanol as internal standard.

RNA extraction and gene expression analysis. Approximately, 100 mg of leaf discs were ground to powder in liquid nitrogen. RNA was isolated according to the manufacturer’s instructions using the Spectrum Plant Total RNA Kit (Sigma-Aldrich, DE). Total RNA quality was checked on RNA ScreenTape using an Agilent 2200 TapeStation (Agilent Technologies, CA).

RNA-Seq and gene expression analysis. cDNA libraries were constructed using 1 µg of total RNA and the KAPA Stranded mRNA-Seq Kit (Kapa Biosystems, MA). Each library was barcoded using the SeqCap Adapter Kit A and B (NimbleGen, Roche, CA) and the target range of fragment sizes (250–280 bp) was confirmed on High Sensitivity D1000 ScreenTape using the Tapestation 2200 (Agilent Technologies, CA). All the libraries were quantified with a KAPA Library Quantification Kit (Kapa Biosystems, MA) using a LightCycler 480 (Roche, DE) and multiplexed in four equimolar pools. Each library pool was sequenced on an Illumina HiSeq 2500 sequencer generating 2 × 50 bp paired-end reads. An average of 54.4 million paired-end reads was generated per sample. Base calling and quality control were performed using the Illumina RTA sequence analysis
pipeline. Reads were aligned to the grapevine PN40024 reference transcriptome\textsuperscript{59,78} using Bowtie2\textsuperscript{79}, obtaining an average rate of read alignment of 84.7%.

Use of RNA-Seq data for the description of the genetic makeup of the genotype under-study. We also used RNA-Seq reads from all samples and stages to perform allele-specific read alignment against a modified version of the grapevine genome reference that included the reference Rpv3-PN40024 haplotype and the alternative resistance Rpv3-1 haplotype and called variant sites using the procedure described in\textsuperscript{18}. RNA reads revealed a substantial number of homozygous sites in transcribed genes across the entire genome and also a number of heterozygous sites in both the Rpv3-1 and the alternative resistance Rpv3-3 haplotype, which are present in a comprehensive diversity-panel of \textit{vitis} as described in\textsuperscript{84} and plotted the density of residual (non-\textit{vinifera}) SNPs in 100-Kb genomic windows. We generated evidence that the introgression in the upper arm of chromosome 14 extends from the upper telomere down to a genomic window whose distal edge is located at the chromosomal coordinate chr14:11,683,740 (Supplementary Fig. S7), approximately 2 Mb downstream of the reported location of the Rpv12 locus\textsuperscript{81}. A closer inspection into the introgression around the Rpv12 locus was conducted using available DNA sequencing reads of ‘Merlot Khorus’, a variety known to carry the Rpv12 resistance haplotype, which derived from the backcross to \textit{vinifer} of a full-sibling of the genotype under study (uncle–nephew relationship), and UD-21076\textsuperscript{84}, which derived from the selfing of ‘Bianca’, the second parent of the genotype under study (Supplementary Fig. S8). We could extract evidence from the nearly complete sharing of non-\textit{vinifera} SNPs between ‘Merlot Khorus’ and the genotype under study (Supplementary Fig. S8e) that they indeed share the same Asian introgression across a large part of the upper arm, including the Rpv12 locus (Supplementary Fig. S8a–e). The resistance chromosome recombined with the \textit{vinifer} counterpart at 11,7 Mbp in the megaspore mother cell of SK77–4/5 that formed the gamete transmitted to the genotype under study. We could also confirm that the homologous chromosome was donated to the genotype under study by ‘Bianca’, because this homolog shared with UD–21,076 exactly the same recombination break points at two introgressed regions (Supplementary Fig. S8b–e). One of these introgressions extended from 5.8 to 11 Mbp. As a result of this, the individual under study is heterozygous for an Asian (Rpv12 resistance haplotype) and an American introgression across approximately 5 Mbp that also include the Rpv12 locus (Supplementary Fig. S8c). Since we did not detect any significant QTL effect on \textit{P. viticola} resistance on chromosome 14 in ‘Bianca’\textsuperscript{12}, which is heterozygous for the American introgression and a \textit{vinifer} haplotype across this locus, we infer that this particular haplotypic combination occurred by chance and was not a target of intentional selection in this individual. We also confirmed that the genotype under study carries two \textit{vinifer} homologous copies along the entire chromosomes 18 and 9 (Supplementary Fig. S7) and it did not show any signal of introgression in or around the reported locations of the Rpv3 locus\textsuperscript{80} and the Rpv10\textsuperscript{80} locus on those chromosomes.

qPCR validation. cDNA was generated using Invitrogen SuperScript IV VILO Master Mix (ThermoFisher Scientific, DE) and 1.5 µg of total RNA. A set of 9 DEGs representative of different GO categories were selected for primer design and validation (Supplementary Table S3). Quantitative PCR reactions were performed using a LightCycler480 thermocycler (Roche, CH) and a 2 x qPCR Bio SyGreen Kit (PCR Biosystems, UK) following the manufacturer’s instructions. Transcripts were quantified using the LC480 software (Roche, CH) and the $2^{-\Delta\Delta C_{T}}$ method using the glyceraldehyde-3-phosphate dehydrogenase (GADPH) housekeeping gene VIT_17s0000g10430 for normalisation with the following primer sequences (forward 5′→3′ TTCTCGTTG AGGGCTATTCACA; reverse 5′→3′ CCACAGACTTCCATCGGTGACA).

Data analysis and graphical representation. Missing data points were replaced with a random value between zero and the limit of quantification of each metabolite. A log$_{10}$-transformation of metabolite concentration was performed prior to statistical analysis\textsuperscript{13}. The t-statistic was computed using the R package Stats\textsuperscript{82}. Metabolite networks were generated using ggraph\textsuperscript{83}. DEG analysis was performed with DeSeq\textsuperscript{83}. Gene enrichment analysis was performed using blast2GO\textsuperscript{86}. Gene functional annotation was retrieved from VitisNet\textsuperscript{86}, VitisCyc\textsuperscript{86}, and V2.1 annotation\textsuperscript{88}. A two-sided Wilcoxon–Mann–Whitney test was used for assessing significant differences between distributions of values. Multivariate data analysis was performed using the R package FactoMineR\textsuperscript{89} and the related graphs in Figs. 1 and 6 were generated using the R package Factoextra\textsuperscript{89}. The R package omicade4\textsuperscript{48} was used to perform MGIA and to generate the plot reported in Fig. 8. Graphs in all other figures were generated with R (version 3.3)\textsuperscript{85}. Metabolite concentrations of Rpv3-mediated defence response were obtained from raw data of our previous paper\textsuperscript{10}.

Data availability RNA-Seq reads were deposited in the European Nucleotide Archive repository under the Unique Name Experiment ERX2989847. Metabolite data are provided as Supplementary Table S1. ‘Merlot Khorus’ FASTQ files of the reads aligning to chromosome 14 are publicly available at figshare under the https://doi.org/10.6084/m9.figsh are.12240881.

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Author contributions
G.C., A.V., M.S., G.D.G. and U.V. designed the experiment. M.S. provided the plant material. G.C., G.D.G., L.Z., and A.V. performed inoculation and sampling. G.C. performed metabolomic analyses. M.P., G.M., S.F. and S.L. performed RNA-Seq analysis and qPCR validation. S.R., A.C. and P. carried out statistical analyses. G.M., S.F., M.M., and G.D.G performed genotypic and haplotypic analyses on the genetic constitution of the genotype under study. G.C., S.F., M.M., G.D.G., and U.V. wrote the manuscript. All authors interpreted the results and commented on the manuscript.

Competing interests
G.D.G. and M.M. are inventors of five patented varieties carrying \textit{Rpv12}-mediated resistance. All other authors declare no potential conflict of interest. Funding bodies had no role in the design of the study and in the collection, analysis and interpretation of data, or writing the manuscript.

Additional information
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