RESEARCH ARTICLE

Rpv3–1 mediated resistance to grapevine downy mildew is associated with specific host transcriptional responses and the accumulation of stilbenes

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Abstract

Background: European grapevine cultivars (Vitis vinifera spp.) are highly susceptible to the downy mildew pathogen Plasmopara viticola. Breeding of resistant V. vinifera cultivars is a promising strategy to reduce the impact of disease management. Most cultivars that have been bred for resistance to downy mildew, rely on resistance mediated by the Rpv3 (Resistance to P. viticola) locus. However, despite the extensive use of this locus, little is known about the mechanism of Rpv3-mediated resistance.

Results: In this study, Rpv3-mediated defense responses were investigated in Rpv3+ and Rpv3ˉ grapevine cultivars following inoculation with two distinct P. viticola isolates avrRpv3+ and avrRpv3–, with the latter being able to overcome Rpv3 resistance. Based on comparative microscopic, metabolomic and transcriptomic analyses, our results show that the Rpv3–1-mediated resistance is associated with a defense mechanism that triggers synthesis of fungi-toxic stilbenes and programmed cell death (PCD), resulting in reduced but not suppressed pathogen growth and development. Functional annotation of the encoded protein sequence of genes significantly upregulated during the Rpv3–1-mediated defense response revealed putative roles in pathogen recognition, signal transduction and defense responses.

Conclusion: This study used histochemical, transcriptomic and metabolomic analyses of Rpv3+ and susceptible cultivars inoculated with avirulent and virulent P. viticola isolates to investigate mechanism underlying the Rpv3–1-mediated defense response. We demonstrated a strong correlation between the expressions of stilbene biosynthesis related genes, the accumulation of fungi-toxic stilbenes, pathogen growth inhibition and PCD.

Keywords: Disease resistance, Downy mildew, Grapevine, Plasmopara viticola, Stilbenes, Vitis vinifera, Metabolomics, Rpv3

Background

The biotrophic pathogen Plasmopara viticola (Berk. & M.A. Curtis) Berl. & de Toni causes grapevine downy mildew, one of the most prevalent grapevine diseases worldwide, leading to significant reductions in berry yield and quality [1]. Due to the lack of genetic resistance of Vitis vinifera species to downy mildew infection, wine production is heavily dependent on the use of fungicides to control this disease. To reduce the dependence of viticulture on chemical inputs, and thereby reduce the ecological and economic burden of wine production, a number of breeding programs have introgressed resistance loci from wild North American and Asian Vitis species into V. vinifera resulting in new downy mildew resistant grapevine cultivars [2, 3]. To date, 27 quantitative trait loci (QTL) conferring resistance to downy mildew have been identified within wild Vitis species [3–8]. However, to date, only the Rpv1 resistance gene from Muscadinia
rotation has been cloned and functionally characterized. Rpv1 is a NB-LRR receptor, involved in pathogen recognition and signal transduction during the initiation of plant defense [9]. Although 27 QTL regions associated with resistance against downy mildew are known, most downy mildew resistant cultivars grown in Europe rely on a single major resistance locus designated Rpv3 (Resistance to P. viticola). The Rpv3-locus was first identified in V. vinifera cv. ‘Regent’ and described in more detail in V. vinifera cv. ‘Bianca’ [10–13]. Other new cultivars with Rpv10 or Rpv12-mediated downy mildew resistance have been generated but are cultivated to a much lower extent. Further characterization of the previously identified Rpv3 locus revealed allelic forms of this locus that all mediate resistance to downy mildew, referred to as Rpv3–1, Rpv3–2 and Rpv3–3 [13–15]. The Rpv3-mediated resistance is associated with the occurrence of necrotic lesions 48 to 72 h post inoculation (hpi), limited mycelial growth and a reduced number of new sporangiophores and sporangia [10, 12, 13, 16]. The cultivar ‘Regent’ (Rpv3–1) is a success story of resistance breeding and is one of the most cultivated downy mildew resistant varieties in Europe [17, 18]. However, despite the widespread use of the Rpv3 resistance locus, detailed knowledge of the underlying mechanism of Rpv3-mediated resistance remains mostly unknown. Understanding the mechanism of resistance mediated by different resistance loci is essential for modern breeding strategies, as the combination of different resistance mechanisms in new grapevine cultivars could reduce the likelihood of breakdown of resistance by the pathogen [19]. Indeed, several studies have shown that P. viticola isolates have arisen in Europe that are able to overcome resistance mediated by the Rpv3 locus [20–22]. In order to establish a successful colonization of grapevine leaves or berries P. viticola must suppress host plant defense mechanisms. It was demonstrated for different oomycetes that this suppression was achieved by the secretion of effector proteins [23] and a general model of plant defense was proposed by Dangl and Jones [24]. The detection of specific pathogen associated molecular patterns (PAMPs) by host pathogen recognition receptors (PRRs) leads to PAMP-triggered immunity (PTI) which is able to prevent non-adapted pathogens from successfully colonizing the plant and causing disease. However, host-adapted pathogens secrete effectors, which suppress PTI, leading to a compatible plant-pathogen interaction and host susceptibility (virulent pathogen isolates). During an incompatible plant-pathogen interaction, caused by avirulent pathogen isolates, these effectors are directly or indirectly recognized by specific resistance proteins with nucleotide-binding domains and leucine rich repeats (NB-LRR) resulting in a transcriptional activation of a variety of defense genes and a resistance of the plant to the pathogen (ETI; effector-triggered immunity) [25, 26]. Successful pathogen recognition leads to activation of signal transduction pathways involving MAP kinases and WRKY transcription factors, which in turn trigger primary immune responses such as accumulation of pathogenesis related (PR) proteins, reactive oxygen species (ROS) or phytoalexins, resulting in a hypersensitive response (HR) that prevents pathogen growth and development [27]. It has been demonstrated for different model organisms that a localized HR at the infection site is a common defense mechanisms observed during ETI [28, 29]. A clear distinction of the mechanisms underlying PTI and ETI cannot be made for all plant-pathogen interactions and some studies indicate overlaps of the defense response elicited by PTI and ETI [29]. For example, in Arabidopsis thaliana, the proteins involved in glucosinolate metabolism AtPEN2 and AtPEN3 are crucial to PTI and ETI [30–32]. It was also shown that degradation products of indole-glucosinolates, whose synthesis is mostly restricted to the order of Brassicales, were involved in ETI-mediated HR [32]. However, it remains unclear if other bioactive secondary metabolites, could play a comparable role in plant defense in other plant species. For example, it has been proposed that stilbenes, which are secondary metabolites in grapevine, may play a similar role in grapevine defense [33]. The stilbene trans-resveratrol is the basic precursor from which all stilbenes found in grapevine are derived and is thus one of the most important stilbenes produced during plant defense [34, 35]. Various modifications of resveratrol result in the generation of bioactive derivatives including ε-viniferins (via oxidative dimerization) or trans-pterostilbene (via methylation). Previous studies have demonstrated the fungicidal and pesticidal effects of these stilbenes on P. viticola sporangia and zoospores [36–38]. In contrast, the glycosylated form of resveratrol, trans-piceid, was found to have only a very limited fungicidal effect on P. viticola sporangia or zoospores [37]. The induction of stilbene synthesis by various biotic and abiotic stresses such as inoculation with Botrytis cinerea or P. viticola or UV-C irradiation was observed in several grapevine varieties [39–43]. Furthermore, a number of previous studies have implicated a role for stilbene biosynthesis in resistance conferred by major R loci originating from wild North American and Asian grapevine species. For example, microarray analysis of the downy mildew resistant species Vitis riparia cv. Gloire de Montpellier revealed a multitude of VvSTS genes to be much more highly induced 12–24 hpi than in comparison to a susceptible V. vinifera cultivar [44]. Boso et al. [45] also observed much higher levels of stilbenes in V. riparia cv Gloire de Montpellier after downy mildew infection compared to V. vinifera. Correlations between resistance against P. viticola and high levels of ε-viniferin and trans-pterostilbene were also demonstrated for Muscadinia rotundifolia genotypes and
an Rpv10-locus containing cultivar [46, 47].

Despite these previous publications implicating a role for stilbene biosynthesis in R-loci mediated resistance, not much is known about their role in Rpv3–1-mediated defense. In this study we have employed a novel approach to investigate this question by comparing downy mildew-induced stilbene biosynthesis, not only between different Rpv3+ and Rpv3– isolates that are either virulent or avirulent on Rpv3 genotypes. Our unique approach provides evidence that the Rpv3-mediated defense response involves the induction of the biosynthesis of fungal toxic stilbenes, resulting in reduced, but not completely suppressed, pathogen growth and development.

Results

The Plasmopara viticola isolate avrRpv3+ overcomes Rpv3-mediated grapevine resistance

Downy mildew resistant grapevine cultivars containing the Rpv3-locus (‘Regent’ and ‘Cabernet Blanc’ - Rpv3–1 and ‘Calardis Blanc’ - Rpv3–1 & 3–2) and the susceptible cultivar ‘Müller-Thurgau’ were inoculated with P. viticola isolates avrRpv3+ and avrRpv3– to evaluate differences in host resistance against the two pathogen isolates. Resistance was assessed by observing the number of sporangia produced 6 days post inoculation (dpi) and the formation of necrotic lesions. After inoculation with the avrRpv3+ isolate, the number of sporangia produced on Rpv3+ cultivars was significantly lower (94–98% reduction) than that observed on the susceptible (Rpv3–) cultivar (Fig. 1a-d, i). In contrast to the susceptible cultivar, necrotic areas were observed on the leaf discs of the Rpv3+ genotypes inoculated with the avrRpv3+ isolate (Fig. 1a-d). No necrotic spots were observed on leaf discs of any genotypes following inoculation with the avrRpv3– isolate (Fig. 1e-h). The amount of avrRpv3+ sporangia was significantly higher in all Rpv3 cultivars compared to the amount quantified after inoculation with the avrRpv3+ isolate, showing that the avrRpv3+ isolate is able to overcome Rpv3-mediated resistance. While, there was no statistically significant difference in the number of sporangia produced by the avrRpv3+ isolate across the different genotypes, the results strongly suggest a reduced susceptibility in ‘Calardis Blanc’ which contains both Rpv3–1 & 3–2 compared to Rpv3–1 only cultivars. For further studies ‘Regent’ was chosen as the representative Rpv3–1 genotype (hereafter designated the Rpv3–1 cultivar).

Rpv3–1-mediated defense responses to avirulent and virulent P. viticola isolates

For histochemical analysis of Rpv3–1-mediated host resistance and pathogen development, the Rpv3–1 and susceptible cultivars were inoculated with the avrRpv3+ and avrRpv3– P. viticola isolates and samples collected 24, 48 and 72 h post inoculation (hpi). Leaf discs were stained with aniline blue to monitor the time course of P. viticola development (Fig. 2). No differences were observed in the early colonization phase between cultivars or between P. viticola isolates. By microscopically observations comparable zoospore attachment to stomata, germ tube development, formation of primary hyphae and development of haustoria were observed in all treatments at 24 hpi (Fig. 2a-d, Additional file 1). At 48 hpi, mycelial growth of the avrRpv3+ isolate was markedly impaired in the Rpv3–1 cultivar, compared to the susceptible cultivars (Fig. 2e–h). Comparing the number of sporangia produced across genotypes inoculated with the avrRpv3+ isolate, the number of sporangia was significantly higher in all Rpv3+ genotypes inoculated with the avrRpv3+ isolate across the different genotypes, the results strongly suggest a reduced susceptibility in ‘Calardis Blanc’ which contains both Rpv3–1 & 3–2 compared to Rpv3–1 only cultivars. For further studies ‘Regent’ was chosen as the representative Rpv3–1 genotype (hereafter designated the Rpv3–1 cultivar).
cultivar (Fig. 2e, f). However, growth of the \textit{avrRpv3}$^-$ iso-
late was similar within the intercellular spaces of the sus-
ceptible and \textit{Rpv3–1} cultivars (Fig. 2g, h). At 72 hpi, the
spongy mesophyll of the susceptible cultivar was entirely
colonized by the mycelium and sporangiophores had been
produced by both isolates, signifying a successful pathogen
life cycle (Fig. 2i, k). In contrast, only weak mycelial
growth and no sporangiophore formation was observed
after 72 hpi for the \textit{avrRpv3}$^+$ isolate on the \textit{Rpv3–1}
cultivar (Fig. 2j), whereas growth and sporulation of the
\textit{avrRpv3}$^-$ isolate on the \textit{Rpv3–1} cultivar was similar to that
observed on the susceptible cultivar (Fig. 2k, l).

In addition to examine pathogen development, the oc-
currence of host programed cell death (PCD) at infec-
tion sites was also examined using trypan blue staining
(Fig. 3). Even though this staining method was optimized
for visualization of PCD, some \textit{P. viticola} structures were
co-stained allowing the identification of infected sto-
mata. At 24 hpi with the \textit{avrRpv3}$^+$ isolate, encysted zoo-
spores were present at stomata of both cultivars, but no
trypan blue-stained cells were visible, indicating that
PCD had not been initiated (Fig. 3a, b). At 32 hpi, PCD
was clearly visible in mesophyll cells below the infected
stomata in the \textit{Rpv3–1} cultivar inoculated with the
\textit{avrRpv3}$^+$ isolate, but no PCD was observed in the sus-
ceptible cultivar (Fig. 3c, d). In addition, no PCD was
observed in any leaf disc of the susceptible or \textit{Rpv3–1}
cultivars up to 48 hpi with the \textit{avrRpv3}$^-$ isolate (Fig. 3e,
f). This histochemical analysis indicate that the \textit{Rpv3–1}-
mediated defense results in restriction of pathogen
growth and development that initiates later than 24 hpi
and is effective before 48 hpi with PCD at 32 hpi.

Expression of stilbene biosynthesis genes correlates with
stilbene accumulation after \textit{Plasmopara viticola} infection
in \textit{Rpv3–1} cultivar

To gain insights into the possible role of stilbene path-
way genes in \textit{Rpv3–1}-mediated resistance, the expression
profiles of a number of different genes involved in stil-
bene biosynthesis were studied by qPCR in the suscep-
tible and \textit{Rpv3–1} cultivars after inoculation with the two
\textit{P. viticola} isolates (\textit{avrRpv3}$^+$ & \textit{avrRpv3}$^-$) or water. Gene
expression was calculated relative to the water controls
and normalized against grapevine housekeeping genes.
One primer set (\textit{VvSTS25/27/29}) was used to quantify
the combined transcript levels of \textit{VvSTS25}, \textit{VvSTS27} and
\textit{VvSTS29}, encoding for putative stilbene synthases,
which have been shown previously to be highly respon-
sive to biotic and abiotic stress [48]. Additionally the
transcript level of \textit{VvROMT}, which encodes a resveratrol
O-methyltransferase catalyzing trans-pterostilbene bio-
synthesis [38, 49], was also analyzed. Transcription of
\textit{VvSTS} and \textit{VvROMT} genes were found to be strongly
up-regulated, within the first 24 hpi, in grapevine tissues
undergoing a resistance response (Rpv3−1/avrRpv3+) when compared to tissues undergoing susceptible interactions (i.e. susceptible/avrRpv3+ and Rpv3−1/avrRpv3−) (Fig. 4). The successful induction of resistance in the Rpv3−1 cultivar inoculated with the avrRpv3+ isolate was associated with a peak of VvSTS and VvROMT transcription at 8 and 12 hpi, respectively. In contrast, the expression of these genes in the Rpv3−1 cultivar inoculated with virulent avrRpv3− isolate or in the susceptible cultivar was relatively constant and lower across the entire infection time course. For example, a clear induction was measured for VvSTS25/27/29 (17 fold) and VvROMT in the Rpv3−1 cultivar inoculated with virulent avrRpv3+ isolate or in the susceptible cultivar was relatively constant and lower across the entire infection time course. For example, a clear induction was measured for VvSTS25/27/29 (17 fold) and VvROMT (14 fold) in the Rpv3−1 cultivar at 8 hpi inoculated with avrRpv3+ compared to leaf discs inoculated with avrRpv3− (Fig. 4). Having demonstrated a significant induction of stilbene biosynthesis pathway genes associated with grapevine leaf tissue undergoing an Rpv3−1-mediated defense response, the next step was to investigate whether this translated into significant differences in the levels and diversity of stilbene compounds within the tissues undergoing avirulent and virulent interactions.

**Activation of Rpv3−1-mediated defense is associated with induction of stilbene biosynthesis**

The level of the four stilbene compounds trans-resveratrol, ε-viniferin, trans-pterostilbene and trans-piceid was determined by HPLC over a 72 h period. Commencing at 24 hpi the successful induction of Rpv3−1-mediated defense response against the avrRpv3+ isolate is associated with a significant higher level of trans-resveratrol, when compared to the infection of the Rpv3−1 cultivar with avrRpv3− isolate and the susceptible cultivar with avrRpv3+ or water controls (Fig. 5). The accumulation of trans-resveratrol, the precursor molecule for stilbenes like trans-piceid, ε-viniferin or trans-pterostilbene was about six fold induced in a successful pathogen recognition and plant defense (Rpv3−1/avrRpv3+), when comparing 6 and 24 hpi (Fig. 5a). This resulted in a significant higher amount of resveratrol at 24 hpi (~2.180 ng g−1

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**Fig. 3** Induction of programmed cell death at the Plasmopara viticola infection site. Leaf discs of a susceptible cultivar (a, c, e) and an Rpv3−1 cultivar (b, d, f) were inoculated with the avirulent (avrRpv3+) P. viticola isolate and samples were taken at 24 hpi (a, b) and 32 hpi (c, d). Leaf discs were inoculated with the virulent (avrRpv3−) P. viticola isolate and samples were collected at 48 hpi (e, f). Abbreviations: st, stomata; z, encysted zoospore; ph, primary hyphae; asterisks indicate trypan blue-stained cells undergoing PCD in response to P. viticola infection. Images are representative of three biological replicates. Scale bars correspond to 50 μm.
FW). In contrast, the level of trans-resveratrol detected in corresponding Rpv3–1 samples inoculated with avrRpv3– or water at 6 and 24 hpi did not change markedly (Fig. 5a) The amount of trans-resveratrol in Rpv3–1/avrRpv3+ samples further increased to 9.000 ng g⁻¹ FW at 72 hpi, resulting in a significantly higher amount of resveratrol during successful defense compared to the inoculated susceptible cultivar (~ 1.600 ng g⁻¹ FW) or Rpv3–1 cultivar inoculated with avrRpv3– (~ 1.500 ng g⁻¹ FW), respectively (Fig. 5a). Trans-resveratrol was also detected in corresponding water controls showing a significant lower amount compared to corresponding time points of the Rpv3–1/avrRpv3– treatment. In contrast, no significant differences were found when comparing the corresponding time points of water controls, with Rpv3–1/avrRpv3– treated samples or susceptible samples (Fig. 5a). Of particular interest was the finding that the two most fungi-toxic stilbenes, ε-viniferin and trans-pterostilbene only accumulated during a successful defense at 48 and 72 hpi, resulting in approximately 800 ng g⁻¹ FW trans-pterostilbene and 12000 ng g⁻¹ FW ε-viniferin at 72 hpi (Fig. 5b, c). A small amount of ε-viniferin (~ 180 ng g⁻¹ FW) was also detected in samples inoculated with the avrRpv3– isolate at 72 hpi, but this was approximately 70 fold lower than the amount found during in leaf tissues undergoing a successful defense response (Rpv3–1/avrRpv3–). The stilbene trans-piceid is the glycosylated form of trans-resveratrol and is considered as transport and storage form of stilbenes without fungi-toxic effects on P. viticola [37]. Trans-piceid was found in all samples, independent of time point, treatment and cultivar indicating that the concentration of this stilbene might not be
important for a successful defense against *P. viticola* (Additional file 2). These results indicate that the significantly higher accumulation of *trans*-resveratrol and the specific biosynthesis of the fungi-toxic stilbenes *ε*-viniferin and *trans*-pterostilbene in *Rpvi3–1* cultivar are associated with the successful activation of the *Rpvi3–1*-mediated defense mechanism in grapevine leaf tissues against *P. viticola*.

**Transcriptomic analysis of differentially expressed genes in response to *Plasmopara viticola* infection**

Gene expression analysis of selected *VvSTS* genes showed the highest induction during a successful *Rpvi3–1*-mediated defense between 6 hpi and 8 hpi (Fig. 4). In order to try and identify other genes involved in the *Rpvi3–1*-mediated defense response, a non-targeted approach was employed using RNA-Seq analysis to identify differentially expressed genes in susceptible and *Rpvi3–1* cultivars 6 hpi with *P. viticola* isolates *avrRpvi3* or *avrRpvi3* or water control (H₂O). RNA-Seq data was first analyzed using a simple pairwise comparison method to identify genes that exhibit a significant differential expression in response to *P. viticola* infection when compared to mock-treated samples of each cultivar. Statistical analysis identified 2612 genes that were differentially expressed with respect to the mock control in at least one pairwise comparison, based on a false discovery rate (FDR) < 10% (multiple adjusted p value *P* < 0.1) and a minimum log fold-change (logFC) of 1. RNA-Seq results were validated by qPCR analysis (Additional file 4) for the *P. viticola* induced genes *VvPR10.1, VvPR5, VvROMT* and *VvSTS1.*
The results demonstrated a strong significant correlation 
\( r = 0.95 - 0.99 \) between qPCR and RNA-Seq data (Additional file 4). Among those 2612 differentially expressed (DE) genes, 34 were found to encode stilbene synthase proteins (Fig. 6a). Of these 34 VvSTS genes, 26 were identified as being more highly induced in leaf tissues undergoing a successful Rpv3–1-mediated defense (Rpv3–1/avrRpv3*) and include the VvSTS genes shown to be up-regulated by qPCR in Fig. 4. Even though most of the individual VvSTS genes are statistically significantly induced by infection in all samples, a significant difference was found when comparing the gene expression of all 34 VvSTS genes (Fig. 6b). This revealed that the total VvSTS expression in Rpv3–1/avrRpv3* was significantly higher compared to susceptible and Rpv3–1/avrRpv3 samples (Fig. 6b). The pairwise comparison method applied above is only able to identify DE genes between inoculated and mock samples of either the susceptible or the Rpv3–1 cultivar but is not suitable to identify genes whose response to infection is statistically significant different between the cultivars. In order to identify candidates differentially expressed during a successful Rpv3–1-mediated defense response, differential expression analysis was performed using linear modelling including interaction terms. These interaction terms made it possible to identify DE genes between the Rpv3–1/avrRpv3* samples and the susceptible/avrRpv3* samples (= successful defense) as well as genes that are differentially expressed between the Rpv3–1/avrRpv3* samples and the susceptible/avrRpv3* samples (= unsuccessful defense). This analysis revealed a total of 2042 DE genes and a Venn diagram was drawn to show the overlap between these two comparisons (Fig. 7, Additional file 5). A total of 85 genes were found to be common between the successful and the unsuccessful defense responses indicating that these genes were differentially expressed in Rpv3–1 samples independent from P. viticola isolates. The analysis indicates that 11 genes specifically expressed in samples undergoing a successful pathogen recognition and defense (Rpv3–1/avrRpv3*), whereas 1946 genes were found to be differentially regulated in Rpv3–1 samples inoculated with the virulent isolate (Fig. 7). This group of 11 genes are of special interest for further studies as they are differentially expressed only during the early stages (6 hpi) of a successful Rpv3–1-mediated defense response. Functional annotation of the encoded protein sequences of the 11 genes in this group showed them to have predicted putative functions as aspartyl proteases (VIT_04s0008g07150; VIT_04s0008g07250), peroxidase (VIT_12s0055g01000), metal-nicotianamine transporter (VIT_16s0098g01250), lipase (VIT_10s0003g02120) and chitinase (VIT_05s0062g01320), a MUTL protein homolog (VIT_04s0044g00170), a Zinc knuckle family protein (VIT_05s0020g01000), a Leucine Rich Repeat receptor-like kinase (VIT_12s0034g02570) and two unknown proteins (VIT_18s0001g07610; VIT_14s0060g02120) (Table 1).

**Discussion**

Histological evaluation of Rpv3-mediated resistance in response to virulent and avirulent P. viticola isolates

In this study, the mechanism of Rpv3-mediated resistance against P. viticola was evaluated by comparing the
induction of defense responses of susceptible and Rpv3 resistant grapevine cultivars after inoculation with avirulent (avrRpv3\(^{-}\)) or virulent (avrRpv3\(^{+}\)) P. viticola isolates. The results revealed that Rpv3-mediated resistance relies on inducible responses specifically elicited by the avirulent (avrRpv3\(^{-}\)) strain, resulting in necrotic lesions and reduced sporulation (Fig. 1), which has been previously described for other P. viticola isolates that are virulent and avirulent on Rpv3 genotypes [16, 20]. Aniline blue staining revealed that zoospores from both isolates were able to encyst at stomata and developed primary hyphae in a comparable manner on both the Rpv3\(^{-}\) and the susceptible cultivars (Fig. 2, Additional file 1). These results are consistent with the previous findings of Kortekamp et al. [50] and indicate that Rpv3\(^{-}\)-mediated resistance relies on inducible responses presumably provoked by the first interaction of plant cells and pathogen hyphae rather than on constitutive defense mechanisms [44]. One of the most studied localized plant response upon pathogen recognition is PCD, which is visible as necrotic lesions at the infection site [51]. The presence of necrotic lesions within 2–10 days after P. viticola infection has been described for several resistant grapevine genotypes with different levels of resistance and it has been speculated whether these differences could be explained by differences in the speed of initiation of PCD which effectively denies the biotrophic oomycete pathogen of nutrition [12, 21, 42, 52, 53]. To our knowledge, this is the first study, presenting a detailed evaluation of the timing of occurrence of downy mildew-triggered PCD in a resistant grapevine genotype. The first differences between a successful and an unsuccessful Rpv3\(^{-}\)-mediated defense response were observed at 32 hpi with the induction cell death, which was followed by inhibition of mycelial growth in the Rpv3\(^{-}\) cultivar inoculated with the avirulent P. viticola isolate (Figs. 2-3). A clear difference in pathogen development was observed at 48 hpi, which resulted in marked reduction, but not complete suppression, of downy mildew sporulation all Rpv3\(^{-}\) cultivars examined (Fig. 1). As grapevines with different origins and Rpv-loci, display a wide range of resistance levels [54], time course studies of PCD progression across these host species could lead to a better understanding of differences in resistance mechanisms and importance of the temporal onset of PCD on P. viticola development in these genotypes. A number of different P. viticola isolates have previously been identified that were able to overcome Rpv3-mediated resistance, demonstrating that the durability conferred by a single resistance locus can be low [16, 20, 22, 55]. The emergence of resistance-breaking pathogens in resistant crops is a well described process during which pathogens can become virulent by evolution of their avirulence genes. As a consequence, resistance proteins are no longer able to recognize these altered avirulent proteins (effectors) [24]. Resistance-breaking isolates develop due to the selection pressure, exerted by plant resistance genes and have been observed in a multitude of crops such as potato and rice [56, 57]. The avrRpv3\(^{+}\) P. viticola isolate we describe is capable of breaking Rpv3\(^{-}\)-mediated resistance (Figs. 1, 2, 3) suggesting that mutated avirulence protein (avrRpv3) is not recognized by the corresponding R gene product of the Rpv3\(^{-}\) locus. The amount of new sporangia produced by this virulent isolate was significantly higher in all Rpv3 cultivars compared to the avirulent isolate. However, Regent (Rpv3\(^{-}\)) and Calarids Blanc (Rpv3\(^{-}\) & 3–2) show differences in mean amount of sporangia when compared to Cabernet blanc (Rpv3\(^{-}\)), which could hint at an elevated level of resistance against the virulent isolate mediated by the presence of additional minor loci [10, 11]. However, it is clear that the avrRpv3\(^{+}\) isolate used in this study was still able to overcome the resistance mediated by Rpv3\(^{-}\) and Rpv3\(^{-}\), suggesting that these two R loci may recognize the same avr effector. Further experiments with genotypes
containing Rpv3–2, in the absence of Rpv3–1, are required to determine whether the Rpv3–2-mediated resistance is also compromised by this avrRpv3– isolate. The combination (pyramiding) of different R loci is recognized as an important strategy to increase durability of resistance against plant pathogens [2, 58]. An understanding of the mechanisms underlying R gene mediated resistance and the recognized avr effectors will be crucial role in finding successful resistance loci combinations to guarantee a durable resistance against grapevine downy mildew.

### Stilbenes and their role in the Rpv3–1-mediated defense

The induction of secondary metabolites in response to biotic and abiotic stresses is a well-known defense reaction. In grapevine, stilbenes are a class of stress-induced secondary metabolites that are commonly involved in responses to various biotic and abiotic stresses [33, 36, 39, 42, 46]. Stilbene synthase (STS) represent the first committed enzyme step in the biosynthesis of stilbenes catalyzing the synthesis of resveratrol [43, 59, 60]. In grapevine, the VvSTS family consists of forty-eight putative VvSTS gene sequences with at least thirty-three full-length sequences encoding potentially functional proteins [43].

Using qPCR analysis it was possible to evaluate the expression level of different VvSTS genes within the first 48 hpi with downy mildew (Fig. 4). The qPCR analysis revealed that these genes were expressed on a comparable and relatively constant level in the compatible interactions (Rpv3–1/avrRpv3– & susceptible/avrRpv3+) over the 48 h infection period and were associated with the accumulation of trans-resveratrol and the non-toxic trans-piceid (Fig. 5). In contrast, VvSTS genes were highly induced within 6–8 hpi in the incompatible (Rpv3–1/avrRpv3+) interaction which resulted in a successful defense response (Fig. 4). This was further confirmed by RNA-Seq analysis of leaf tissue sampled at 6 hpi which confirmed an elevated level of transcription in a total of 34 VvSTS genes in the incompatible interaction (Fig. 6). However, interaction term analysis showed that the direction of regulation and strength is not statistically different between Rpv3–1/avrRpv3– and Rpv3–1/avrRpv3+ samples when compared to susceptible plants which indicates the induction of VvSTS genes in general is not specific to a successful defense. Still, when looking at the overall fold changes across the whole set of VvSTS genes (Fig. 6b), it was demonstrated that during a successful defense (in Rpv3–1/avrRpv3+ samples) a network of VvSTS genes is upregulated even further than in susceptible samples. It can be speculated that this results in synthesizing a higher level of trans-resveratrol that provides the precursors for additional biosynthetic reactions leading to the production of the oligomeric stilbenes ε-viniferin and trans-pterostilbene. Gene expression analysis of the resveratrol O-methyltransferase (VIT_12s0028g01880), a gene that encodes a protein responsible for the biosynthesis of trans-pterostilbene from resveratrol [38] also revealed a higher level of expression in leaf tissues undergoing a successful Rpv3–1-mediated defense response (Fig. 4 for qPCR, Additional file 3 for RNA-Seq) compared to susceptible samples. Thus, the expression data of stilbene biosynthesis-related genes shows a strong correlation with the detectable levels of stilbene compounds (Fig. 5).

The toxicity of the different stilbenes on sporangia or zoospores of P. viticola has been previously investigated [36, 61]. These studies showed that trans-piceid had no toxicity and trans-resveratrol only low toxicity on P.
**viticola** sporangia and zoospores. In contrast, ε-viniferin and trans-pterostilbene were found to have strong fungitoxic effects on grapevine downy mildew. However, conclusive evidence of a direct role for stilbenes in reducing the susceptibility of certain grapevine genotypes to **P. viticola** infection is still lacking. It has previously been shown that whereas the stilbenes trans-resveratrol and trans-piceid may be induced in both susceptible and downy mildew-resistant cultivars, the fungi-toxic oligomeric forms (ε-viniferin and trans-pterostilbene) are found exclusively, or at much higher levels, in downy mildew-resistant cultivars [35, 42, 46, 47, 62]. Similarly, our results show that ε-viniferin and trans-pterostilbene were detected exclusively in leaf discs displaying a successful defense response against the avirulent **P. viticola** isolate (Fig. 4) strongly suggesting a role for these compounds in **Rpv3–1**-mediated defense. While trans-resveratrol has only low toxicity to **P. viticola**, it may have another role in **Rpv3–1**-mediated defense other than as a precursor of viniferin and trans-pterostilbene biosynthesis. Chang et al. [63] showed that the addition of exogenous trans-resveratrol inhibited the growth of **Vitis** cell suspension cultures and activated defense-related responses such as ROS formation and cell death. They postulated that trans-resveratrol could itself act as a signaling molecule initiating PCD. Interestingly, we observed the first cells undergoing PCD in **Rpv3–1** genotypes at 32 hpi (Fig. 3), not long after the appearance of elevated levels of trans-resveratrol (Fig. 5). Vezzulli et al. [14] recently demonstrated a correlation between **Rpv3–3** locus-mediated resistance against downy mildew and the induction of oligomeric stilbenes in a ‘Mezling’ x ‘Teroldego’ segregating population. They postulated that downy mildew resistance in this population was likely mediated by the combined action of the **Rpv3–3** locus and stilbene biosynthesis. The results presented here complement their findings by showing that induction of stilbene biosynthesis pathway genes and the accumulation of oligomeric fungitoxic stilbenes are specifically upregulated following recognition of the **avrRpv3** effector by **Rpv3–1** and are likely to be an important component of **Rpv3**-mediated defense. Ultimately, conclusive proof of a role for stilbenes in **Rpv3**-mediated resistance can only be obtained by studying the downy mildew resistance of **Rpv3** genotypes in which the stilbene synthase gene family has been deleted or silenced which would be particularly challenging given the large number of **VvSTS** genes in the grapevine genome [43].

**Early specific transcriptomic responses of the **Rpv3–1**-mediated resistance mechanism**

The first transcriptional defense responses of **Rpv3–1** cultivar ‘Regent’ have been reported between 6 and 8 hpi here and in other studies [64, 65]. In order to discover other transcriptional and biochemical pathways, in addition to the stilbene biosynthesis pathway that might be involved in **Rpv3–1**-mediated downy mildew resistance we also compared early (6 hpi) transcriptomic responses of leaf tissues undergoing compatible and incompatible interactions with **P. viticola**. Evaluation of **Rpv3–1**-mediated transcriptional responses by RNA-Seq analysis confirmed the induction of a large number of host genes in both interactions, although this occurs for a number of genes with greater intensity in the incompatible interaction [42, 64]. However, it is difficult to draw any conclusions from these results because of the influences of genomic background of the host plants on differences in gene expression cannot be excluded. Most transcriptional studies that set out to identify genes specifically involved in **R** gene-mediated resistance are based on comparisons of gene expression between a resistant genotype that contains the **R** gene and a susceptible genotype that doesn’t. However, the analysis in this case is complicated by differences in gene expression arising from the different genetic backgrounds of the host species. Therefore, our approach was to not only compare the transcriptional responses of susceptible and resistant genotypes, but also use an **Rpv3** resistance-breaking **P. viticola** isolate (avr**Rpv3**') to compare transcriptomic response of the same **Rpv3–1** cultivar undergoing a successful and unsuccessful defense. In order to analyze the RNA-Seq data comprehensively, statistics was done in two parts. In a first statistical approach, RNA-Seq data was analyzed using pairwise comparisons between **P. viticola** and mock treated samples in order to identify DE genes in response to **P. viticola** infection irrespective of the genotypes. Secondly, a more sophisticated statistical approach using linear modeling including interaction terms was performed in order to compare the differential gene expression responses in the **Rpv3–1/avrRpv3** samples compared to susceptible/avr**Rpv3** samples (= successful defense) and the differential gene expression responses in **Rpv3–1/avrRpv3** samples compared the susceptible/avr**Rpv3** samples (= unsuccessful defense). In the first approach using simple pairwise comparisons a total of 2612 genes were DE with respect to the mock control in at least one pairwise comparison. Using the second more stringent statistical approach many genes were excluded whose DE is influenced by events unrelated to **Rpv3–1**-mediated defense mechanism. In total 2042 DE were found in this approach. Interestingly, only one of the previously identified 34 STS genes which showed different expression in the pairwise comparison (infected vs mock) was DE comparing the different genotypes (interaction term analyses). This is in line with results depicted in Fig. 4 showing a positive regulation for STS genes upon treatment irrespective of genotype and the accumulation of trans-piceid in all treatments (Additional file 2). As discussed before (chapter 3.2), despite the general induction
of VvSTS genes in response to an infection, the overall fold changes across the whole set of VvSTS genes (Fig. 6b) during a successful defense (in Rpv3–1/avrRpv3+ samples) is significantly up-regulated at 6 hpi compared to susceptible samples (Fig. 6b). This could result in synthesizing a higher level of trans-resveratrol that provides the precursors for additional biosynthetic reactions of fungi-toxic oligomeric stilbenes during the Rpv3–1-mediated defense response. These genes might therefore not be specific markers for a successful defense while 11 of those 2042 genes were found to be DE specifically in Rpv3–1/avrRpv3+ samples (Fig. 7) when compared to susceptible/avrRpv3+ samples. These 11 genes could only partially be detected using the pairwise statistical approach and they might provide interesting putative marker genes for plants undergoing a successful defense (Table 1). Of these 11 genes, one was functionally characterized as a class III plant peroxidase (VIT_12s0055g01000). Plant peroxidases play a crucial role in many physiological processes and especially in plant defense. Indeed, it was recently demonstrated that peroxidase genes underlay a QTL region that contributes to Rpv3–3-mediated resistance to downy mildew [14]. Moreover, it has been suggested that peroxidases are able to catalyze the synthesis of ε-viniferin, which was exclusively detected in this study in samples undergoing a successful defense (Fig. 5) [34]. Peroxidases also represent an important class of pathogenesis-related proteins that are able to limit pathogen growth by catalyzing lignification of cell wall components or by producing reactive oxygen species that are involved in hypersensitive response [66, 67]. Two other genes that were found to be differentially expressed in Rpv3–1/avrRpv3+ samples were functionally characterized as α- and αβ-aspartyl protease (VIT_04s0008g07150, VIT_04s0008g07250). Even though the role of aspartyl proteases in plants is still hypothetical, some studies have postulated a possible involvement of aspartyl proteases in PCD and autophagocytosis in response to fungal infection [68, 69]. Another DE gene in this group was identified as a GDSL lipase (VIT_10s0003g02120). The physical and molecular functions of GDSL esterases/lipases genes in grapevines are not yet known, but they have been reported to play a role in morphogenesis, plant development, synthesis of secondary metabolites, and plant defense response in other plant species [70, 71]. Moreover a metal-nicotianamine transporter YSL3 (VIT_16s0098g01250) and a chitinase (VIT_05s0062g01320) were found in the group of the 11 DE genes. Chitinases are known pathogen-related proteins playing a role during plant defense even though oomycetes are a less likely target for chitinases, due to the almost absence of chitin in this group of pathogens [72, 73]. In conclusion, the RNA-Seq analysis based on comparative gene expression in an Rpv3–1 genotype inoculated with virulent and avirulent P. viticola isolates has identified genes which might be specifically involved in the early stages of Rpv3–1-mediated plant defense and which will be the subject of more detailed examination to determine their putative role in Rpv3–1 resistance against P. viticola.

Conclusions
Histochemical, transcriptomic and metabolomic analyses of Rpv3+ and susceptible cultivars inoculated with avirulent and virulent P. viticola isolates were performed in this work to investigate mechanism underlying the Rpv3–1-mediated resistance response. We demonstrated a strong correlation between the expressions of stilbene biosynthesis related genes, the accumulation of fungi-toxic stilbenes, pathogen growth inhibition and programmed cell death. Our results indicate that pyramiding different Rpv3 loci can increase the level of resistance to an avirulent downy mildew isolate but seems not enhance durability of resistance against virulent isolates. Furthermore, several candidate genes potentially involved in Rpv3-mediated resistance against P. viticola were identified, which will be further studied to unravel the mechanism of resistance.

Methods

Plant material, Plasmopara viticola isolates and leaf disc infection
Potted grapevines were grown under greenhouse conditions (22 °C/day, 18 °C/night; 50% humidity). Vitis vinifera cv. ‘Müller-Thurgau’; ‘Regent’ (Rpv3–1) [74], ‘Calardis blanc’ (Rpv3–1, Rpv3–2) [15] and ‘Cabernet blanc’ (Rpv3–1) (unpublished data) were regenerated from canes obtained from the State Education and Research Center of Viticulture, Horticulture and Rural Development, Neustadt/Weinstr. Germany as described previously [65]. The plant material of this study has been identified and certified by Mr. Nesen (Agricultural chamber of Palatinate, Neustadt, Germany) and is deposited in the herbarium of the Julius Kühn-Institut (Bundesforschungsinstut für Kulturpflanzen, Geilweilerhof, Siebeldingen, Germany). A P. viticola isolate that is virulent on Rpv3 genotypes was originally collected from a commercial ‘Cabernet blanc’ vineyard, whereas an isolate that is avirulent on Rpv3 genotypes was collected on a susceptible cultivar in Rhineland-Palatinate (Germany) in 2016. According to the classification used previously by Casagrande et al. [16], these isolates were designated avrRpv3+ (avirulent) and avrRpv3– (virulent), based on their ability to trigger (or not) cell death on Rpv3 grapevine genotypes. Isolates were further propagated as described by Malacarne et al. [42]. For all infection experiments, leaf discs (1.5 cm diameter) were excised with a cork borer from the fourth or fifth fully expanded leaves below the shoot apex. Leaf discs were placed upside down on filter paper soaked with 4 ml distilled water
(dH₂O) in a 92 mm diameter petri dish. Freshly harvested sporangia were placed into dH₂O to release zoospores that were used for inoculation. Four droplets of the zoospore suspension (10 μl each with 40000 sporangia ml⁻¹) or sterile dH₂O (mock) were placed on the abaxial leaf surface. Droplets were removed with paper 12 h post-inoculation (hpi). Petri dishes were sealed with parafilm and incubated at 22 °C with a photoperiod of 16 h light / 8 h dark until sampling occurred. To reduce the potential contribution of the leaf disc wound surface to changes in gene transcription and metabolite levels, leaf discs were recut (1.3 cm diameter) to remove the outer 2 mm wounded edge, immediately prior to freezing in liquid nitrogen.

Phenotypic evaluation of resistance to *Plasmopara viticola* isolates

For each treatment, a total of 40 leaf disks were cut from leaves sampled from four individual plant replicates and randomly distributed onto petri dishes prior to inoculation. The development of necrotic lesions was macroscopically scored at 6 days post inoculation (dpi). Additionally, the degree of *P. viticola* infection was quantified by counting the number of sporangia produced per leaf disc at 6 dpi accordingly to Merz et al. [65]. The average of three independent experiments is shown. Averages of each experiment were used for the statistical analysis.

Histochemical studies

Aniline blue staining was used to monitor *P. viticola* mycelium development according to Hood and Shew [75]. Leaf discs were inoculated with zoospore suspensions as described above. Samples were collected at 24, 48 and 72 hpi and documented with an epifluorescence microscope (ZEISS Axio Scope.A1; Kübler HXP-120C lighting device; Filter set: Zeiss 05; software AxioVision Rel. 4.8). Programmed cell death was studied by trypan blue staining at 24, 28, 32 and 48 hpi as described in Feechan et al. [76]. For a photographic record of leaf disc tissues a ZEISS Axio Lab.A1 microscope with a Zeiss AxioCam MRc camera and Zen blue software were used.

Determination of stilbene content

Five individual plant replicates of ‘Müller-Thurgau’ and ‘Regent’ were sampled to obtain leaf disks. Each biological replicate was distributed onto a petri dish prior to inoculation. At each time point two leaf discs per replicate and treatment were pooled together and collected for RNA extraction, obtaining 10 leaf discs at each time point and treatment. For RNA-Seq analysis an additional experiment with five individual plant replicates was performed to obtain at 6 hpi a second replicate. Leaf discs inoculated with *P. viticola* isolates or treated with H₂O (mock) were collected at 6, 8, 10, 12, 24 and 48 hpi. Total RNA was isolated with the Spectrum Plant Total RNA purification kit (Sigma Aldrich), following the manufacturer’s instructions and used for qPCR and RNA-Seq analysis. RNA purity (A260/A280 nm) and quantification were measured using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific Inc, Wilmington, DE, USA). A qPCR reaction on crude RNA was performed, showing no gDNA contamination.

Quantitative real time PCR expression analyses

For cDNA synthesis, 350 ng of grapevine total RNA was reverse transcribed using the dART cDNA synthesis kit (Roboklon) as described in Höll et al. [48]. Transcript analysis of genes of interest (GOI) during *P. viticola* infection were determined by qPCR with the SYBR Green method on a Rotor-Gene Q (Qiagen). The PCR reaction mix (15 μl) contained cDNA (1.2 ng), primer (10 μM each),
dNTP mix (10 mM each) (Sigma Aldrich), JumpSTART polymerase (2.5 U/µl) (Sigma Aldrich), 0.15 µl from 1:40 dilution SYBR Green in H₂O (ABSolute™ QPCR SYBR® Green Fluorescein Mix; 1:10 in DMSO; ABgene) and nuclelease free water. The thermal cycling conditions used were 95 °C for 6 min followed by 40 cycles of 95 °C for 15 s, 58 °C for 30 s, and 72 °C for 20 s, followed by a melt cycle with 1 °C increments (5 s) from 56 to 96 °C. The primer efficiency was tested with cDNA dilutions of samples. Normalization against the reference genes VvUbiquitin, VvEF1a and VvGAPDH [78] was conducted as described by Pfaffl et al. [79]. The Rotor-Gene Q Series Software Q 2.0.2 (Qiagen) and the Q-Gene software [80] were used for analyzing melt curves and measurement of primer pair efficiency. Gene-specific oligonucleotide sequences are shown in Additional file 6 and representative melting curves are presented in Additional file 7.

RNA-Seq analysis
Preparation of RNA-Seq libraries
For RNA-Seq analysis at 6 hpi, two individual experiments were performed, obtaining two replicates for each treatment with 10 leaf discs pooled from five individual plants for each replicate. Leaf discs of susceptible cultivar ‘Müller-Thurgau’ were inoculated with the avirulent P. viticola isolate (avrRpv3+) or water. Leaf discs of the partially resistant cultivar ‘Regent’ (Rpv3–I locus) were inoculated with avrRpv3+ or avrRpv3– isolates or water. From these five experimental conditions, two biological replicates were used for RNA extraction and sequencing library construction resulting in ten samples for RNA-Seq analysis. The quality of the extracted total RNA that was used for library construction was checked with the Agilent 2100 Bioanalyzer (Agilent Technologies) and only RNA samples with an RNA integrity number > 7 were used for library preparation. Libraries for next generation sequencing were prepared using the NEBNext Ultra II Directional RNA Preparation Kit with NEBNext Dual Index Oligo’s for Illumina and the NEBNext Poly A Selection Module (New England Biolabs) according to the manufacturer’s instructions, at the Bioquant, CellNetworks Deep Sequencing Core Facility (Heidelberg, Germany). Single-end sequencing with a length of 75 bp for each read was run on an Illumina NextSeq 500 instrument at the Genomics Core Facility, EMBL (Heidelberg Germany). After sequencing, raw data were transferred to the Quantitative Biology Center (QBiC, https://portal.qbic.uni-tuebingen.de/portal/) at the University of Tübingen using an Aspera client.

Quality control, mapping, and differential expression analysis
Initial steps from raw data quality control to mapping and eventually read counting was undertaken by QBiC on the High Performance cluster (HPC) of the University of Tübingen using a fully automated workflow written in Snakemake [81]. The code is accessible here: https://github.com/qbicsoftware/rnaseq. This workflow utilizes the following software packages: FastQC (version 0.11.4) for initial raw data quality control, Cutadapt (version 1.8.3) for filtering reads containing matches to Illumina adapters, Tophat (version 2.2.3.0) for mapping of filtered reads against the reference genome and HTseq-count (version 0.6.1p2) for counting. In the mapping step, reads were aligned to the Vitis vinifera reference genome PN40024 [82] downloaded from Ensembl Plants (annotation release 38) in January 2018. Differential expression (DE) analysis was performed using the R packages limma (version 3.32.10) and edgeR (version 3.18.1). First, the raw read count table was filtered for genes that had no expression in any of the samples. Then the remaining counts were normalized by sequencing depth and log2-transformed using functions in edgeR to meet the assumptions of linear models. In order to identify differentially expressed genes in P. viticola infected versus mock treated samples with respect to susceptibility given by the genetic background (susceptible versus Rpv3–I), a linear model was fitted to each gene consisting of a fixed effect for a combined factor of genotype (susceptible versus Rpv3–I) and treatment (inoculated with avrRpv3+ or avrRpv3– versus control). This combination of the two main experimental conditions into one factor allowed the extraction of simple contrasts of interest (e.g. susceptible-infected (avrRpv3+) versus susceptible-control). The same approach allowed the extraction of more complex interaction terms such as [Rpv3–I_infected (avrRpv3+) versus Rpv3–I_control] versus [susceptible_infected (avrRpv3+) versus susceptible_control] in order to identify which genes respond to infection differently concerning different cultivars. The simple pairwise contrasts as well as more complex interaction terms were extracted from the same statistical model applied to the same dataset. Limma was also used to calculate empirical Bayes moderated p-values relative to a minimum required fold-change threshold which were adjusted for multiple testing by controlling the false discovery rate (FDR) ≤0.1% [83].

Additional files

Additional file 1: Plasmopora viticola infection at 24 h post inoculation on leaves of susceptible and Rpv3–I cultivars. Germinated sporangia were visualized by UV epifluorescence after aniline blue staining. P. viticola spores of the avirulent (avrRpv3+) isolate on the (A) susceptible grapevine cultivar and on (B) Rpv3–I cultivar and of the virulent (avrRpv3–) P. viticola isolate on (C) susceptible grapevine cultivar and (D) Rpv3–I cultivar are shown. Images are representative of three biological replicates. Scale bars correspond to 50 µm. (TIFF 369 kb)
Additional file 2: Amount of trans-piceid produced in response to Plasmopara viticola inoculation. Trans-piceid was measured in a susceptible and an Rpv3−1 cultivar after inoculation with P. viticola isolates (avrRpv3−1 or avrRpv3+) or treatment with water (H2O). Samples were collected 0, 6, 24, 48 and 72 hpi. Each bar represents the mean of four biological replicates. Bars represent the average of one experiment with four biological replicates and two independent measurements. Error bars show standard deviation. ANOVA was used to determine the effect of cultivar and treatment (the two isolates) on the stilbene amount and then means were compared by Tukey’s HSD test. Statistical analysis is related to significance of all samples at the same time point, different letters (a, b, c) are significantly different (P < 0.05). (TIFF 105 kb)

Additional file 3: Pairwise comparison analysis using LIMMA of Vitis vinifera gene expression in response to Plasmopara viticola infection. Blue font indicates significant up-regulation, while red font highlights significant down-regulation (adjusted P ≤ 0.1). Gray font denotes genes with fold changes that were not significant (adjusted P > 0.1). FC, fold change. (XLSX 460 kb)

Additional file 4: Comparison of RNA-Seq and real-time qPCR analyses. Scatterplot of the correlation between normalized counts (P. viticola vs mock) of four expressed genes (VvPR10.1, VvPR5, VvROMT and VvSTS1) as assessed by RNA-Seq analysis and the relative expression levels (fold-change relative to the expression in control plants and normalized against housekeeping genes) as assessed by qPCR. (A) Rpv3−1 (avrRpv3−1 vs mock), (B) susceptible (avrRpv3+ vs mock) and (C) Rpv3−1 (avrRpv3−1 vs mock). A linear trend is shown. (TIFF 93 kb)

Additional file 5: List of 2042 differentially expressed genes identified using interaction term analysis and displayed in Venn diagram (Fig. 7). xInteractor term analysis terms to identify DEG characteristic for a successful defence by comparing the pairwise contrasts of Rpv3−1 samples with susceptible samples. (adjusted P ≤ 0.1). FC, fold change. (XLSX 305 kb)

Additional file 6: Sequence of the oligonucleotides used for qPCR analysis. (XLS 9 kb)

Additional file 7: Melting curves of oligonucleotides used for qPCR analysis. Description of data: Pictures show a representative melting curve of a cDNA template (red) and the negative control (light blue) of (A) VvEF1a, (B) VvGAPDH, (C) VvUbiquitin, (D) VvSTS25/27/29, (E) VvROMT, (F) VvPR10.1, (G) VvPR5 and (H) VvSTS1. x axis shows the temperature (°C) and y axis the change in fluorescence level with respect to temperature increase (ΔF/ΔT). (TIF 12742 kb)

Abbreviations

avvRpv3−1: Virulent Plasmopara viticola isolate; avvRpv3+: Avirulent Plasmopara viticola isolate; DE: Differentially expressed; dpi: Days post inoculation; ETL: Effector-triggered immunity; hpi: Hours post inoculation; HR: Hypersensitive response; PAMPs: Pathogen associated molecular patterns; PCD: Programmed cell death; QTL: Quantitative trait loci; ROS: Reactive oxygen species; Rpv: Resistance to Plasmopara viticola

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Authors’ contributions

BE contributed to project concept, conceptualized the experiments, conducted the experiments, analyzed the data and drafted the manuscript. SC performed the RNA-Seq analysis and contributed to the manuscript revision. TZ carried out the metabolite analysis and contributed to the manuscript revision. OT carried out QTL analysis of Cabernet Blanc and contributed to the manuscript revision. GB supported microscopy studies. AK and TR contributed to project concept and manuscript revision. JB conceptualized as well as coordinated the project, and revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

Data from this project was also deposited in Gene Expression Omnibus (GEO) of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/geo/) with the following accession number: GSE128865.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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