Received: 14 October 2020 | Accepted: 27 April 2021

DOI: 10.1111/ppa.13404

Plant Pathology WILEY

ORIGINAL ARTICLE

Defence responses of grapevine cultivars to powdery mildew: Ontogenic resistance versus genetic resistance

Agnes Calonnec[1] | Jerome Jolivet[1] | Marie-Louisa Ramaroson[1,2] | Marie-Cecile Dufour[1] | Marie-France Corio-Costet[1]

[1]UMR SAVE Santé et Agroecologie du Vignoble, INRAE, Bordeaux Sciences Agro, Univ. Bordeaux, Villenave d'Ornon, France

[2]INRAE, UMR IRHS, Beaucouze, 49071, France

Correspondence
Agnes Calonnec, UMR SAVE Santé et Agroecologie du Vignoble, INRAE Bordeaux Sciences Agro, Univ. Bordeaux, 33140 Villenave d'Ornon, France
Email: agnes.calonnec@inrae.fr

Funding information
French Biodiversity Agency (OFB), Grant/Award Number: CASDAR launched by the French Ministrie

Abstract

The most sustainable management of resistant varieties involves understanding which defence mechanisms the plant uses according to its resistance genes and stage of development and how effective these mechanisms are. Three grapevine varieties were compared for their susceptibility versus resistance to powdery mildew: *Vitis vinifera* 'Cabernet Sauvignon' (CS) and two hybrids, 'Artaban' and 'Prior'. Four different leaf ages were collected from 2-year-old grafted grapevines planted in pot or in vineyard, and several variables were assessed for each leaf: (a) morphological and physiological indicators, (b) pathogenicity traits: spore germination, infection efficiency, mycelial growth and sporulation, and (c) gene expression using a high-throughput quantitative reverse transcription PCR method to inform about plant defence status and functioning of primary metabolism. A significant decrease in germination and sporulation **with increasing leaf age** were observed for the three varieties: Prior showed a susceptibility similar to that of CS, whereas Artaban was fully resistant with no sporulation. Genes highlighted as markers of the variety were involved in defence (*VvPR3*, *VvPR4* overexpressed in Artaban, *VvPR4bis*, *VvAlli2* in Prior) or in primary metabolism (repression of *VvCitS* in Artaban, *VvCHI* in Prior, overexpression of *VvCAD2*, *VvGST3* in Prior). After inoculation, more defence genes, especially PR proteins, were overexpressed in the oldest leaves as potential components of ontogenic resistance. PR proteins accounted for 25% of total disease variation. Phenylpropanoid and ethylene pathways were leaf age-dependent. The statistical method used highlighted the contributions of the genotypic expression involved in genetic (cultivar) and ontogenic (leaf age) resistances and of the metabolic pathways to the disease.

KEYWORDS
Artaban, defence genes, grapevine *Vitis*, NeoViGen96, powdery mildew, prior

1 | INTRODUCTION

Erysiphe necator, the grapevine powdery mildew, is an obligate biotrophic fungus that is able to develop sophisticated mechanisms to invade living plant cells, such as up-regulating the expression of cell wall invertase and plant hexose transporter genes or down-regulating vacuolar invertase genes (Hayes et al., 2010). This fungus is a ubiquitous pathogen that affects cultivars of *Vitis vinifera* and is responsible for significant damage worldwide (Bois et al., 2017). Despite the ability of *V. vinifera* to initiate basal defence mechanisms in certain varieties, such as Cabernet Sauvignon (Fung et al., 2008), young leaves are highly susceptible to powdery mildew

(Calonnec et al., 2018). One hypothesis to explain this susceptibility is that effectors released by adapted powdery mildew species combine with the plant's mildew locus O proteins (MLO) to prevent the fungal chitin from being recognized as a pathogen-associated molecular pattern (PAMP) by the plant's membrane receptors and thus suppress PAMP-triggered immunity (PTI) (Dry et al., 2010). The recognition between PAMPs and plant receptors such as LysM receptor kinases (Brulé et al., 2018) activates mitogen protein kinases, which control the synthesis and/or signalling of defence hormones, the activation of defence genes, the synthesis of antimicrobial metabolites, and hypersensitive response (HR)-like cell death (Meng & Zhang, 2013). Inversely, the low susceptibility measured in old leaves could be attributed to an increase in the sugar level, the induction of constitutive defences, or changes in the expression or activity of the *MLO* genes, consequently restoring the PTI and resulting in ontogenic resistance (Calonnec et al., 2018; Qiu et al., 2015).

Cultivated grapevine (*V. vinifera* subsp. *vinifera*) originating from Eurasia has been domesticated and cultivated for approximately 5,000 years. Unfortunately, it carries no resistance genes to its most damaging pathogens, *Erysiphe necator* (causal agent of powdery mildew) and *Plasmopara viticola* (downy mildew), both of which are native to North America. Most *Vitis* species from which resistance genes have been identified have coevolved with powdery mildew in North America. To date, 13 resistance genes to powdery mildew have been identified in wild grapevine species (Merdinoglu et al., 2018). These resistance genes, after recognition of powdery mildew effectors and the restoration of plant defences, should allow a moderate to high protection level against powdery mildew (Qiu et al., 2015). This is what we refer to as genetic resistance as opposed to the ontogenic resistance related to the age of the tissues found in all *Vitis*. The first identified gene, *Run1* (*Resistance to Uncinula necator*), belongs to the TIR-NBS-LRR family of proteins (Feechan et al., 2013). *Vitis* species that have coevolved with powdery mildew have thwarted the pathogen's effectors by developing resistance proteins that are capable of recognizing effectors and restoring plant defences in a process called effector-triggered immunity (ETI). ETI initiates a signalling cascade that leads to transcriptional reprogramming in the host plant and the expression of plant defence genes (Welter et al., 2017). The consequence of this defence is a reduction in sporulation of the pathogen, or even absence of symptoms. The type of interaction is then called incompatible. However, there is limited knowledge about the resistance mechanisms triggered by the various resistance loci (Qiu et al., 2015), and an overlap probably exists with the mechanisms triggered by PTI. The PTI would lead to a moderate level of resistance or total susceptibility (compatible interaction), depending on the defence mechanisms involved.

The use of resistant varieties in viticulture can be a strong lever for controlling epidemics, especially in the context of the rational and sustainable use of pesticides at the European level (European directive 2009/128/EC). At the French national level, the framework of specific Ecophyto plans aims to protect vineyards by drastically reducing phytosanitary treatments by 25% in 2020 and 50% in 2025. Recently, growing of resistant varieties was authorized in France, making it possible to accelerate the use of new innovative systems (Montaigne et al., 2016). However, in addition to problems involving the breakdown of resistance genes for downy as well as powdery mildews (Cadle-Davidson et al., 2011), the effectiveness of certain resistant quantitative trait loci (QTLs) or genes in controlling epidemics can be dependent on the gene considered, genetic background, organs, and plant development (Calonnec et al., 2013). To ensure the sustainability of these new systems, it would be necessary to determine the cultural practices, training systems, climate, and gene associations that make them more or less vulnerable to pathogens. As these factors have an indirect effect on vine physiology, growth, and defence, it would be necessary to define the periods of maximum susceptibility when the crop should be protected or monitored more carefully. Such characterizations are essential in the establishment of varietal ideotypes, that is, varieties that ideally combine traits that are able to enhance yield and quality within a given socioeconomic context and label. Thus, we need to better understand the features implemented by the plant to defend itself, depending on its resistance genes and on its development (ontogenic resistance). Accounting for ontogenic resistance is particularly important for perennial plants such as grapevines that are subjected to pathogen attack, epidemics of which are driven by plant development and cultural practices (Mammeri et al., 2014). We also need tools able to easily assess this defence to better control and optimize the resistance. These are prerequisites if we want to develop resilient agroecosystems at different scales (crop management, use of plant defence stimulator, low input, crop genetic diversity; Peterson et al., 2018).

In this study, we analysed (a) the behaviour (growth, physiology, susceptibility, gene expression) of two resistant varieties and compared it to a susceptible *V. vinifera* 'Cabernet Sauvignon'; (b) the gene expression for the three varieties prior and in response to inoculation with powdery mildew for different leaf ages; and (c) we propose an original statistical method to quantify the contribution of the genotypic expression involved in ontogenic resistance and/or genetic resistance and to highlight the contribution of the metabolic pathways to the disease. The analysed genes were those commonly associated with PTI/ETI responses and gene markers of primary metabolism.

2 | MATERIALS AND METHODS

2.1 | Plant materials

Three cultivars were used in this study: the susceptible *V. vinifera* 'Cabernet Sauvignon' (CS) and the resistant varieties 'Artaban' and 'Prior'. Both resistant varieties were registered in the official French catalogue of wine grape varieties in 2017 (https://info.agriculture.gouv.fr/gedei/site/bo-agri/document_administratif-ba2c6a22-4cc3-450e-aa64-cf59183dbb47). Artaban is part of the InraResDur breeding programme aimed at creating varieties with durable resistance to powdery and downy mildews. It originated from

the cross VRH3082-1-42 × Regent. VRH3082-1-42 is derived from a cross between *Muscadinia rotundifolia* and *V. vinifera* followed by four backcrosses with *V. vinifera*. Regent is a German (JKI institute) cultivar derived from a cross between Chambourcin (Joannes Seyve 11368 × Plantet) × Diana (Sylvaner × Müller Thurgau). Artaban thus carries two genes that confer resistance to powdery mildew, *Run1* and *Ren3*, provided by both parents, and two genes for resistance to downy mildew, *Rpv1* and *Rpv3* (Merdinoglu et al., 2018; Pauquet et al., 2001; Welter et al., 2007). *Run1*/*Rpv1* were derived from *M. rotundifolia*, whereas *Ren3*/*Rpv3* were transmitted by *V. rupestris* and *V. aestivalis*, ancestors of Regent. Artaban, previously scored in the laboratory and in the field, shows complete resistance to powdery mildew and a high level of resistance to downy mildew, whereas Regent shows partial resistance to both pathogens (Calonnec et al., 2013). The cultivar Prior is a German variety resulting from the cross of Freiburg 4-61 × Bronner, which has *V. rupestris* in its ancestry (just like Regent), but also *V. amurensis*, a *Vitis* native to Asia. The cultivar probably carries *Ren3 Ren9*/*Rpv3.1 Rpv3.2* (Vitis International Variety Catalogue; http://www.vivc.de/index.php).

The experiments were conducted in 2017 using leaves from 2-year-old grafted vines planted in 2016, either in 20 L pots filled with a commercial soil mixture (Substrat 4 [267]; Klasmann-Deilmann France) or in the field. The three varieties were used for the pot experiment while the two resistant varieties were used for the field experiment. The varieties were grafted on rootstock 3309C for Artaban and CS and on rootstock Fercal for Prior. Rootstocks were chosen based on the field soil characteristics as well as on the vigour of cultivars and vine management planned in the vineyard. All potted vines were maintained outside at the INRA experimental station Grande Ferrade at Villenave d'Ornon, France (44°47′25.21″N, 0°34′33.41″W) and pruned at one node. Vines were not treated with any fungicide throughout the duration of the experiment. The bud break occurred on average on 17 March (45 growing degree days; between 9 March for Prior and 30 March for CS; Figure S1). Each new fully expanded primary leaf (width from 3 cm) was tagged using a set of plastic colour markers to indicate the date of leaf appearance and subsequently to indicate the leaf age. Field and pots were located 400 m from each other on the INRA experimental station. Environmental conditions before the experiments are indicated in Table 1.

All leaf samples from the pot experiment were used for the phenotypic characterization, bioassays, and gene expression before and after inoculation. The leaf samples from the field experiments were only used for phenotypic characterization and gene expression without inoculation. The gene expression under field and pot growth conditions were further compared for the two resistant varieties.

2.2 | Plant characterization

Measurements of maximum leaf width (W) and length (L) were performed outdoors on each leaf the day before the bioassay. The leaf surface (LS) was subsequently estimated by multiplying W × L by

TABLE 1 Experimental conditions

Experiment	Date of sampling (D)	Climatic conditions before the experiment								Cultivar	No. sampled shoots	Phenological stage (mean no. leaf/shoot ± *SD*)
		During the 10 days before D					At D − 1					
		Average temperature (°C)	Average GD	Sum rain (mm)	Average ETPP	Average global radiance (J/cm^2)	GD	Average temperature (°C)	Sum rain (mm)			
1 (pot)	11 May	14.59	52.2	33.5	3.82	1,367	220.1	15.6	4.5	Cabernet Sauvignon	18	11.33 ± 1.05
										Prior	18	9.66 ± 1.15
										Artaban	18	12.05 ± 0.70
2 (field)	19 May	17.94	76.2	32.5	4.05	495	288.4	13.2	21	Prior	18	10.00 ± 0.94
										Artaban	18	11.50 ± 0.95

Abbreviations: ETPP, evapotranspiration rate; GD, growing degrees, sum of degrees above 10 °C.

a coefficient function of the variety. Coefficients to calculate the leaf surface were determined separately by assessing the surface by image analysis of a set of 30 detached leaves (0.557, 0.638, and 0.612 for CS, Prior, and Artaban, respectively). Measurements of physiological indicators were performed for each leaf using a leaf-clip meter (Dualex Scientific+). The leaf-clip provided a nondestructive measurement of the chlorophyll (Chl) content in units of µg/cm^2 and of in situ epidermal polyphenol, flavonol index (Flav), and anthocyanin (Ant) contents (Cerovic et al., 2012). The results are based on the near-infrared chlorophyll fluorescence measured under a reference excitation induced by red light (650 nm) compared to a sampling light specific for polyphenols (e.g., UV-A 375 nm for flavonols and 530 nm for anthocyanins). The measured Chl:flav ratio can be used to estimate Chl_m/$EPhen_m$ (mass-based chlorophyll content/mass-based epidermal polyphenols) and, by extension, the Prot:Phen ratio (total protein based on the amount of total phenolic compounds) to assess the N and C allocations in tissues (Meyer et al., 2006). Leaves from 18 shoots for each variety were measured for each experiment, the day before sampling (10 May for pot experiment and 18 May for field experiment). In the field, leaves were measured at BBCH 71 for Prior and BBCH 66 for Artaban. Field characterizations are summarized in Figure S2.

All leaf samples used in plant characterization were further used for the bioassays and gene expression as described in the subsequent sections.

2.3 | Bioassays

Before leaf sampling, petioles were marked with a colour code to identify their respective positions on the shoot and to maintain traceability throughout the whole experiment. Leaves aged 6, 10, 15, and 20 days were sampled on 11 May and 19 May 2017 for pot and field experiments, respectively, and immediately transported to the laboratory in a humid chamber (Figure S3). The leaves were detached, washed in water, and then dried on filter paper. Under axenic conditions, the leaves were disinfected in 5% (wt/vol) aqueous calcium hypochlorite at 65% available chlorine for 10 min, rinsed in sterile water, and dried on sterile filter paper. Four leaf discs (22 mm) were cut from each individual leaf and three discs were individually placed abaxial face down in three water agar (WA) plates (20 g/L) supplemented with benzimidazole (30 mg/L). The leaf discs in WA plates were subsequently used for (a) pathogenicity tests: conidia germination and infection efficiency (G-INF) and sporulation (SPO); and (b) gene expression after inoculation (Ex1). Each individual WA plate contained a total of six discs representing three varieties (V) × two leaf ages (LA). The fourth disc was placed in individual tubes and used for gene expression before inoculation (Ex0) (Figure S3). Each Ex0 tube contained three replicate leaf discs from one variety × leaf age. The Ex0 tube were immediately frozen at −80 °C until analysed for gene expression before inoculation. Pathogenicity tests (G-INF and SPO) were based on 18 replicate discs. All WA plates were randomly placed in a settling tower and inoculated by blowing conidia from a leaf infected 10 days earlier with powdery mildew (PM). The density of deposited inoculum was assessed by counting the spores on the agar of a randomly selected Petri dish using a binocular magnifier (988 ± 256 conidia/cm² mean and *SD* between tower assessed on a magnifier's field of view of 0.5 cm). The plates were placed in a growth chamber at 22 °C under a 12:12 hr light:dark photoperiod for pathogen development. At 24 and 48 hr postinoculation (hpi), a tape test was performed for two replicate discs of each treatment combination (V × LA). For this test, a tape was applied to the leaf discs, removed, and stained with cotton blue. The maximum mycelial length (MYC) per germinated spore was measured under an Olympus BX51 stereomicroscope using AnalySIS software. One hundred spores were measured per disc. At 72 hpi, the percentage of spores that germinated or were defined as infectious (assumed to go to the end of the infection cycle) were assessed using the tape test previously described. Conidia that produced at least the primary appressorium (stage 2; Leinhos et al., 1997) were considered germinated (G) whereas those that reached the branching hyphae stage (5–6) were considered infectious (INF). The total production of spores per infected disc was measured 13 days after inoculation, on average 6 days after the end of the latent period for the incubation temperature of 22 °C. Sporulating discs were placed in a vial filled with 20 ml of isotone 2 and one drop of nonionic dispersant (Nacconol 90F; Beckman Coulter) and shaken, and spore production was assessed by counting the number of particles between 15 and 38 µm in diameter in a 500 µl sample using a particle counter (Coulter Counter Multisizer 3; Beckman Coulter). The disease variables were calculated as follows: G, INF (the % of germinated and infectious spores, respectively, as defined above), SPO (number of spores per cm^2 of infected leaf disc), and MYC (average hyphal length per germinated spore; Calonnec et al., 2018). For the pot experiment, 18 vines per variety were sampled at one shoot per plant, while nine vines per variety at two shoots per plant were sampled for the field experiment. For the field experiment, only three discs were sampled per leaf (no Ex1).

2.4 | Gene expression analysis by quantitative reverse transcription PCR

Leaf discs from three biological replicates (identical leaf age and variety, different shoots) were pooled, frozen in liquid nitrogen, and stored at −80 °C. For each variety, leaf age, and inoculation condition, three to four replicates were extracted. After each sample was crushed in liquid nitrogen, total RNA was extracted following the protocol described by Dufour et al. (2016).

Extraction buffer preheated to 56 °C was added to the leaf powder (20 ml/g; 300 mM Tris.HCl, pH 8; 25 mM EDTA; 2 µM NaCl; 2% CTAB; 2% polyvinyl polypyrrolidone (PVPP); 0.05% spermidine trihydrochloride; and 2% β-mercaptoethanol added extemporaneously). The mixture was stirred vigorously and incubated in a water bath at 56 °C for 10 min with regular stirring. An equal volume of chloroform/isoamyl alcohol (24:1 vol/vol) was added and then centrifuged

at 3,500 × g for 15 min. The subsequent steps were conducted using the Spectrum Plant Total RNA kit (Sigma) according to the manufacturer's instructions. Finally, the samples were incubated for 15 min with the DNase I digestion set (Sigma). RNA concentrations were determined with a Denovix DS-11 spectrophotometer. Ten micrograms of total RNA was reverse-transcribed using 2 µM oligo-d$(T)_{15}$, ribonuclease inhibitor, and M-MLV reverse transcriptase (Invitrogen) in a final volume of 50–100 µl according to the manufacturer's instructions. The obtained cDNAs were stored at −20 °C. High-throughput gene expression quantification was conducted using microfluidic dynamic array (Fluidigm) technology. Specific primer sets are included with the NeoViGen96 chip, designed previously by Dufour et al. (2016), which contains genes that are potentially involved in grapevine defence, with five new genes involved in primary metabolism. The chip includes a set of **22** PR proteins, **11** genes involved in the phenylpropanoid and isoprenoid biosynthesis pathways, 13 in cell wall reinforcement, three in the oxylipin pathway, **17** in phytohormone biosynthesis or regulation (salicylic acid [SA], jasmonic acid [JA], and ethylene), five in glutathione S-transferase, and **five** markers involved in the Krebs cycle and photosynthesis (Table S1).

Real-time quantitative PCR (qPCR) was performed using a BioMark HD system (Fluidigm Corporation). The 96.96 dynamic array was used for qPCR, according to the manufacturer's protocol (http://www.fluidigm.com/user-documents). Five microlitres of the mixture was prepared for each sample containing 1× TaqMan Universal Master Mix (without UNG), 1× GE sample loading reagent (Fluidigm PN 85000746), and each diluted preamplified cDNA. The loaded chip was placed in the BioMark system for PCR at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The data were analysed using real-time PCR BioMark 2.0 analysis software (Fluidigm Corporation) for cycle quantification (C_q) and by applying the same principle of classical real-time PCR with the Stratagene MX3005P system where the fluorescence signal of the amplified DNA intersected with the background noise.

The R package HTqPCR (3.10) from Bioconductor and its limmaCtData function (Dvinge & Bertone, 2009) were used. Data are normalized by subtracting the mean of the housekeeping genes from all other values in the feature set, and multiple *t* tests were performed to test statistical significance of C_q values between features and samples to identify genes significantly differentially expressed between varieties, growth conditions, or leaf age.

For the pot experiment, gene expression relative to that of the susceptible control (youngest leaf of CS at 24 hr before or after inoculation) allowed the comparison of features under compatible versus incompatible interactions and to evaluate the effect of genetic versus ontogenic resistance on the disease. Relative expression was calculated based on a multiple gene normalization method using the principles and formulas of Hellemans et al. (2007). The geometric mean of selected housekeeping genes was used as a normalization factor. The differential gene expression obtained for all genes and modalities was averaged and $\log_2$ transformed.

Genes showing differential patterns according to varieties or leaf age were selected for partial least squares path model analysis.

2.5 | Data and statistical analyses

Linear regressions were performed on physiological variables (Chl, Flav, Ant) to test their relationship to leaf age. Slopes were compared (two-tailed) with the null hypothesis that the slopes were identical (Prism 5 v. 5.01). Pathogenicity variables (G, INF, SPO) were analysed by repeated measures analysis of variance (ANOVA) with varieties as the factor and leaf age as the repeated measure. Fisher–Pitman permutation tests were performed to compare physiological variables and pathogenicity variable values two by two between different varieties and leaf age classes. With permutation tests, the validity of the statistic is based on an empirical distribution derived from resampling the data in accordance with the null hypothesis being true. There is therefore no need assumption about the distribution of the data, especially with skewed distributions for certain age groups (package coin of R statistical software v. 3.1.2, function one way-test with approximate distribution; number of Monte Carlo redistribution 9,999). Hyphal lengths (MYC) observed for the three varieties and four leaf ages at two postinoculation times (24 and 48 hpi) were compared by permutation tests.

A heatmap based on Pearson correlation coefficients was generated on the data to visualize the clustering of features and samples at once (plotCtHeatmap function R package HTqPCR). The clustering makes it possible to compare and group together the most similar samples in terms of gene expression without having to choose a reference sample. A pairwise comparison design was given to the limmaCtData function to compare C_q values between samples (varieties or leaf age) by multiple *t* tests for the different features using the Benjamini–Hochberg method at the 5% or 10% significance level. When varieties or leaf age are compared, all repetitions from the same condition versus leaf age are considered.

Nonparametric multiple comparisons were used to compare gene expression of target samples to the reference Cabernet Sauvignon 6 day leaf noninoculated (CS 6d NI) or Cabernet Sauvignon 6 day leaf inoculated (CS 6d I) (package 'nparcomp' and Dunnett's test, 5% significance level).

To measure the relationship between metabolic pathways and disease, and metabolic pathways and leaf age and varieties, on the other hand, a partial least squares path model analysis (PLS-PM) was performed (Tenenhaus et al., 2005). The PLS path model was described by nine unobservable or latent variables (LVs): Varieties, Leaf age, PR proteins, Ethylene SA, Phenylpropanoids, Cell wall reinforcement, Signalling, Primary metabolism, and Disease. The path model was designed to explain the extent to which variation of Disease can be predicted by the variables corresponding to metabolic pathways (six endogenous variables) and to which metabolic pathway variables can be predicted by the exogenous variables Leaf age and Varieties. Each LV was constructed using a set of observable (or measured) variables called manifest variables (MVs). For the metabolic pathway variables, the MVs corresponded to the gene expression values (differential expression $\Delta\Delta C_t$ of data Ex1) of selected genes. Leaf age and Varieties were constructed in a formative way based on qualitative variables (6d,10d,15d, 20d

for leaf age, and CS, Prior, Artaban for varieties). The Disease variable was described by the variables characterizing the three basic pathogen traits: percentage of germinated spores (G), percentage of infectious spores (INF), and amount of sporulation (SPO). The standardized latent variables were estimated as linear combinations of their centred MVs. Collinearity between manifest variables was allowed. The PLS-path model was described by the measurement model, which related the different MVs to their own LVs, and the structural model, which linked the endogenous LV Disease to the other LVs: PR proteins, Ethylene SA, Phenylpropanoids, Cell wall reinforcement, Signalling, and Primary metabolism. The inner estimate was performed using a path weighting scheme algorithm. Confidence intervals for regression coefficients were estimated by bootstrap methods (1,000 random sampling of 35 individuals in the observed data set). Two criteria for the goodness of fit of the model (GoF) were provided to evaluate the external model (relationship between MVs and LVs) and the internal model (relationship between LVs). Altogether, 37 observations (modalities of rep × LA × V) were considered. The analysis was performed using the XLstat PLS-PM module v. 2012.2.02. The analysis was compared to another one with the latent variable Physiology instead of Leaf age. This variable was described by the MVs 'Chl' (chlorophyll), 'Flav' (flavonoids), and '1/Ant' (1/anthocyanin), which corresponded to the measurements with the leaf clip Dualex and 'SA' (leaf surface area). All variables were quantitative.

3 | RESULTS

3.1 | Plant characterization

The hybrid Prior was characterized by the largest leaf surface, especially those grown in the field, and the lowest chlorophyll content (Figure 1a,b). Except for the oldest leaves in the field, the leaves sampled had not reached their maximum size and were still actively growing. The leaf content in Chl increased linearly with leaf age (over the leaf age range considered), and the slopes differed significantly between varieties (F = 17.59, p < 0.001), with the greatest increase observed for both potted and field vines of Artaban (Figure 1b). Chl content was lower in potted plants relative to field vines. The flavonol (Flav) content decreased significantly with leaf age for CS (F = 21.8, p < 0.001; Figure 1c). In contrast, for the two resistant varieties, the Flav content was almost stable with leaf age, except in the youngest leaves of Prior on potted plants ($p_{\text{Prior pot}}$< 0.001, $p_{\text{Prior field}}$ = 0.38, $p_{\text{Artaban pot}}$ = 0.49, $p_{\text{Artaban field}}$ = 0.78). Anthocyanin content decreased in all varieties with leaf age, with lower amounts in the field and the highest amounts for Prior within each condition (Figure 1d). The nitrogen balance index (NBI), measured using Dualex, which provides the Chl:Flav ratio, increased with leaf age (Figure 1e), reflecting a greater investment in photosynthetic proteins than in phenolic compounds for construction of the dry mass of older leaves.

3.2 | Pathogenicity

3.2.1 | Spore germination and mycelial growth

At 72 hpi, the frequency of germinated spores decreased with increasing leaf age for the three varieties (significant leaf age effect p < 0.001) with significant interaction of varieties with leaf age (Figure 2a). The difference between extreme leaf ages was significant for each variety for germination (G), infection efficiency (INF), and mycelium growth (MYC) based on permutation tests (p > 0.99). According to the Fisher–Pitman multiple comparison, germination (G) was not significantly different between CS and Prior, regardless of the age of the leaf (Table 2). For Artaban, only the two oldest leaves (15–20 days) clearly showed a significantly lower germination of spores compared with Prior and CS. When INF was considered, the differences between varieties were enhanced, with Prior being significantly more susceptible than the two other varieties (higher % of ramified spores) on the youngest leaves (6–10 days) while Artaban was significantly less susceptible than CS regardless of leaf age (Figure 2b, Table 2). The hyphal length of germinated spores showed that mycelial growth almost stopped between 24 and 48 hpi for Artaban (except for leaf age 10 days), while the progression almost doubled for CS and Prior (Figure 2c). Similar results were observed for leaves from field vines (data not shown).

3.2.2 | Sporulation

No sporulation was observed on Artaban, and no significant difference in sporulation was observed between CS and Prior, although a strong decrease in sporulation with leaf age was observed for both cultivars (Figure 2d, Table 2).

3.3 | Gene expression

3.3.1 | Effects of growing conditions in gene expression

Few genes were significantly differentially expressed between field and pot conditions for Artaban and only the nitrite reductase (*VvNIR*) was highly expressed significantly in the field for both resistant varieties (Figure 3a). The alliinase *VvAlli2*, the isocitrate dehydrogenase *VvIDH41*, and the glutathione S-transferase *VvGST1* were overexpressed in the field for Prior, whereas thaumatin-like osmotin *VvPR5bis* and *VvGST3* were overexpressed in pots in Artaban (Figure 3b). Six genes could be considered as good variety markers because they were significantly differentially expressed between the two resistant varieties, regardless of the conditions (field versus pot): the PR protein endochitinase *VvPR3* and the chitinase *VvPR4* overexpressed in Artaban and, to a lesser extent, the chalcone isomerase (*VvCHI*) less expressed in Prior; the citrate synthase (*VvCitS*), a gene of the Krebs cycle repressed in

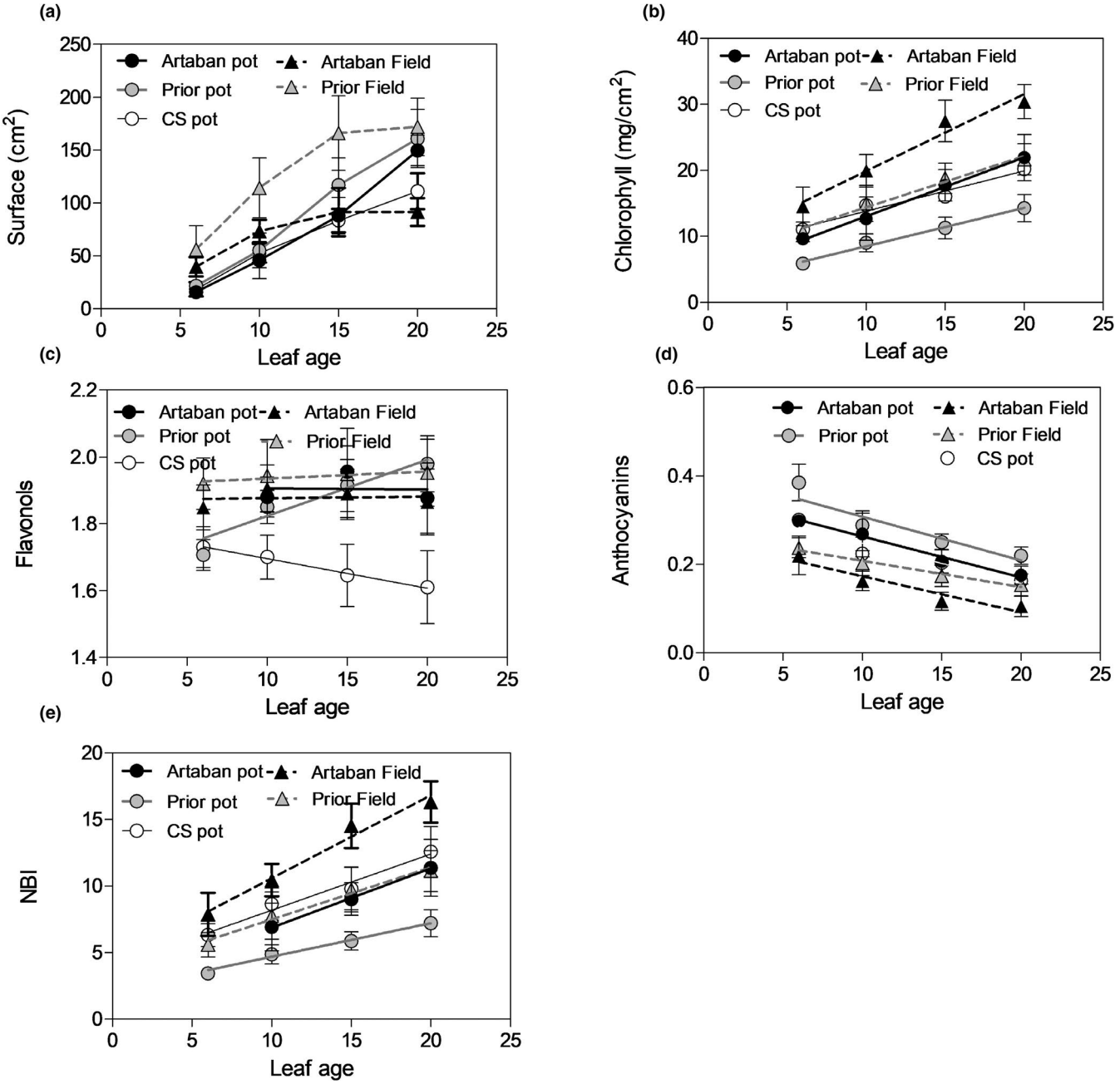

FIGURE 1 Comparison of the three grapevine varieties, Cabernet Sauvignon (CS), Prior, and Artaban, according to their leaf surface (a), chlorophyll (b), flavonols (c), anthocyanins (d), and nitrogen balance index (NBI) (e) as a function of the age of the leaves from 2-year-old grafted grapevines grown in pots or in the field. Bars correspond to the standard deviation. For b, c, d, and e, regression lines are indicated

Artaban (Figure 3b); and the two cinnamoyl alcohol dehydrogenases (*VvCAD* and *VvCAD2*) were moderately expressed in Prior, whereas *VvCAD* was preferred in Artaban (Figure 3b). Other genes had an expression level that varied according to the age of the leaf. They coded for genes involved in different metabolic pathways with either decreasing expression with leaf age for genes involved in cell wall construction, such as callose synthase (*VvCALS3*), pectin methyl esterase (*VvPECT2*), and 3-hydroxy-3-methylglutaryl CoA reductase (*VvHMGR*), potentially involved in cellular multiplication; oxido-reduction status with *VvGST1* and *VvGST4*; or PR protein types, such as proteinase inhibitor (*VvPR6*) and defensin (*VvPR12*). On the other hand, expression increased with leaf age for a few genes involved in defence, such as ACC oxidase (*VvACO1*), phenylalanine ammonia-lyase (*VvPAL*), resveratrol *O*-methyltransferase (*VvROMT*), stilbene synthase (*VvSTS*), or the PR protein PR1, described as a defence marker with a potential function in sterol binding (Gamir et al., 2017) (Figure 3c). As a result of these variations, a hierarchical clustering analysis, based on Pearson correlation on all features with C_q averaged by repetition (field and pot), separates first the youngest leaves (6–10 days) from the oldest (15–20 days), and then within each of the age classes, separates the varieties (Figure S4).

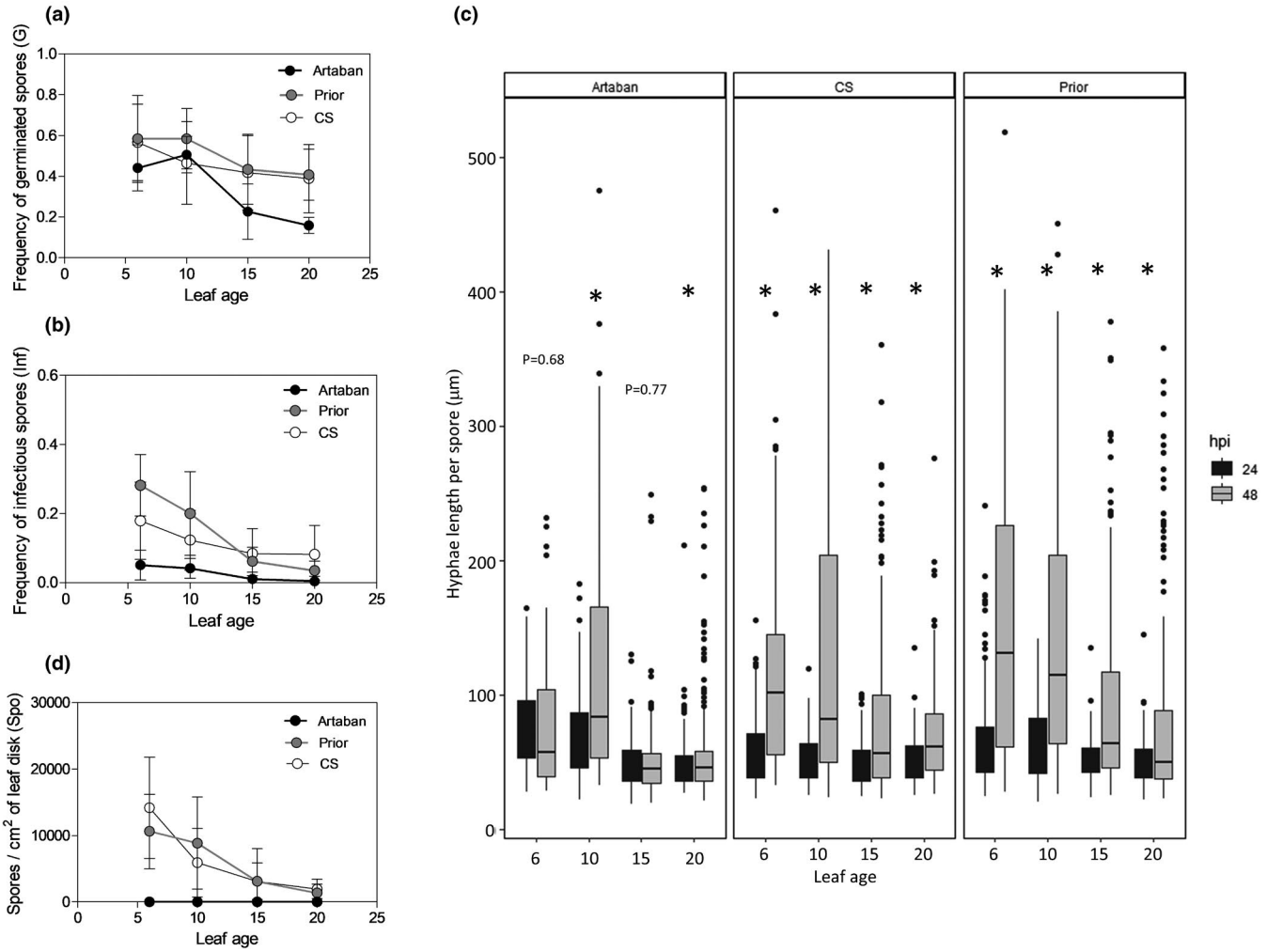

FIGURE 2 Pathogenicity tests based on frequency of germinated spores (a), infectious spores (b), and sporulation (d) as a function of leaf age for the three varieties, Artaban, Prior, and Cabernet Sauvignon (CS), on potted grapevine leaves. Bars indicate standard deviations for the 18 replicates. Box plot of the total hyphal length per spore (c) at 24 hr postinoculation (hpi) and 48 hpi as a function of leaf age (three replicates). Asterisk indicates a significant difference ($p < 0.05$) between 24 and 48 hpi according to permutation tests

3.3.2 | Gene expression before inoculation

In the pot experiment, the gene expression of both resistant varieties was compared to that of the susceptible variety CS, either by calculating the relative expression to its youngest leaf or directly after normalization of C_q values. Again, Artaban significantly overexpressed the PR protein endochitinase *PR3* gene, repressed citrate synthase (*VvCitS*), and overexpressed the chorismate synthase gene *VvCHORS2* (involved in the shikimate pathway leading to the synthesis of aromatic amino acids) and the defensin PR12 protein (Figure 4a, Figure S5). In Prior, signature markers of the variety compared to CS were alliinase (*VvAllii2*; involved in the synthesis of alliicin, recognized for its antifungal activity), cinnamoyl alcohol dehydrogenase (*VvCAD2*; involved in cell wall reinforcement through lignin synthesis), and glutathione S-transferase *VvGST3*, which were overexpressed, and chalcone isomerase (*VvCHI*; involved in the flavonoid pathway), which was repressed (Figure 4b, Figure S5). Four genes were on average more highly expressed in both resistant varieties: a chitinase type V (*VvPR11*), a chitinase type I (*VvPR4bis*), a methyl salicylate esterase (*VvSABP2*), and a glutathione S-transferase *VvGST1* (Figure 4c, Figure S5). Three genes were overexpressed in CS: a chitinase type III (*VvCHIT3*), an ascorbate peroxidase (*VvAPOX2*; an antioxidant enzyme involved in the reduction of H_2O_2 into H_2O), and the PR protein PR1bis (Figure 4d). The expression of these last two genes increased with leaf age. Three other genes were leaf age-dependent: ACC oxidase (*VvACO1*), for which expression increased with leaf age, and lipoxygenase *VvLOX2* and NADH plastoquinone oxidoreductase (*VvNADH*), which decreased with leaf age (Figure 4e).

3.3.3 | Gene expression after inoculation

A summary of the genes of the different metabolic pathways that were overexpressed or repressed after inoculation with PM for each of the three varieties or according to leaf age or both is presented in

TABLE 2 Disease variables (G, INF, SPO) measured for the three grapevine cultivars Cabernet Sauvignon (CS), Prior, and Artaban, and four leaf ages and *p* value for the two sample Fisher–Pitman permutation test comparing varieties for each leaf age

Leaf age	Frequency of germinated spores (G)			*p*			Frequency of infectious spores (INF)			*p*			Spores/cm² leaf disk (SPO)		*p*
	CS	Prior	Artaban	CS–Prior	CS–Artaban	Prior–Artaban	CS	Prior	Artaban	CS–Prior	CS–Artaban	Prior–Artaban	CS	Prior	CS–Prior
6	0.56 a	0.58 a	0.44 b	0.595	0.011	0.993	0.18 a	0.28 a	0.05 c	0.998	<0.001	1	14,161	10,609	0.074
10	0.46 b	0.58 a	0.50 b	0.973	0.781	0.969	0.12 b	0.19 a	0.04 c	0.974	<0.001	1	5,905	8,841	0.905
15	0.41 a	0.43 a	0.22 b	0.620	<0.001	0.999	0.08 a	0.06 a	0.01 b	0.100	<0.001	1	3,099	3,091	0.503
20	0.38 a	0.40 a	0.15 b	0.650	<0.001	1	0.08 a	0.03 b	0.003 c	0.013	<0.001	1	1,945	1,343	0.123

Note: Identical letters following the value within rows indicate no significant difference.

Figure 5. For Artaban, the pattern observed before inoculation was further observed with lower expression of citrate synthase (VcCitS) and substantially increased overexpression of *VvPR3*. In addition, but to a lesser extent, an overexpression of the PR4 protein chitinase type I (*VvPR4*), and significant under-expression of nitrate reductase (*VvNR*) and of the transcription factor *VvWRKY2* were observed (Figure 6, Figure S6). The defensin *VvPR12* was also slightly over-expressed compared to CS (10% significance level). For Prior, the pattern of overexpression of *VvAllii2* and *VvGST3* and lower expression of *VvCHI* was also observed after inoculation. In addition, we observed overexpression of the isocitrate dehydrogenase *VvIDH41* (a gene in the Krebs cycle), and of the two chitinases *VvPR4bis* and *VvPR8*, especially for the oldest leaves, and of the lignin-forming peroxidase (*VvPOX*). There was no additional overexpression of the cinnamoyl alcohol dehydrogenase *VvCAD2* but instead a repression of *VvCAD*. Other genes were slightly up-regulated in both resistant varieties: *VvPR11* and *VvSAPB2a*, as observed before inoculation, and a β-1,3-glucanase (*VvGLU*). For CS, overexpression of *VvAPOX2*, *VvCHIT3*, and *VvPR1bis* was observed, with an overexpression of a serine protease (*VvPIN*), of a β-1,3-glucanase (*VvPR2*), and of the lipoxygenase *VvLOX9* (Figure 6, Figure S6).

The expression of genes also varied with leaf age; several genes showed a pattern in relation to leaf age with repression or overexpression in old leaves for both compatible and incompatible interactions. These patterns include (a) five genes involved in cell wall and plasmalemma reinforcement (*VvCALS3*, *VvPER*, *VvPECT2*, *VvCAGT*, *VvHMGR*) and a lipid transfer protein (*VvPR14bis*) decreased with leaf age; (b) overexpression with increasing leaf age was observed for the main genes in the phenylpropanoid pathway (flavonoids; *VvCHS*, *VvDFR*, *VvF3H*, *VvLDOX*, or stilbenes; *VvPAL*, *VvSTS*), even if these genes were repressed by the pathogen (relative to *CS* 6d uninoculated, data not shown) and for ACC oxidase (*VvACO1*); an increase in expression with leaf age was also measured for the four genes coding for PR proteins CHIT4α (an endochitinase type IV), PGIP (a polygalacturonase inhibiting protein), PR15 (a germin-like protein), and PR5bis (a thaumatin like-osmotin) (Figure 7, Figure S7); and (c) a few other genes involved in primary metabolism or gene regulation increased with leaf age (*VvCAD2*, *VVGST5*, *VvLox2*, *VvNR*, *VvPR1*).

The hierarchical clustering analysis based on the whole set of features with C_q averaged by repetitions (varieties × leaf age) first separated the youngest leaves (6–10 days) from the oldest (15–20 days), and then within each of the age classes, separated the varieties (Figure 8).

3.4 | Relationship between metabolic pathways – varieties – leaf age – disease

The PLSpath model could explain 73% of the variation in the 'Disease' latent variable between samples ($R^2 = 0.73$), with each disease manifest variable having a similar weight (0.34 for SPO, 0.38 for G, 0.36 for INF) (Figure 9). Three metabolic pathways were negatively correlated with the 'Disease', which means that when the expression of the associated

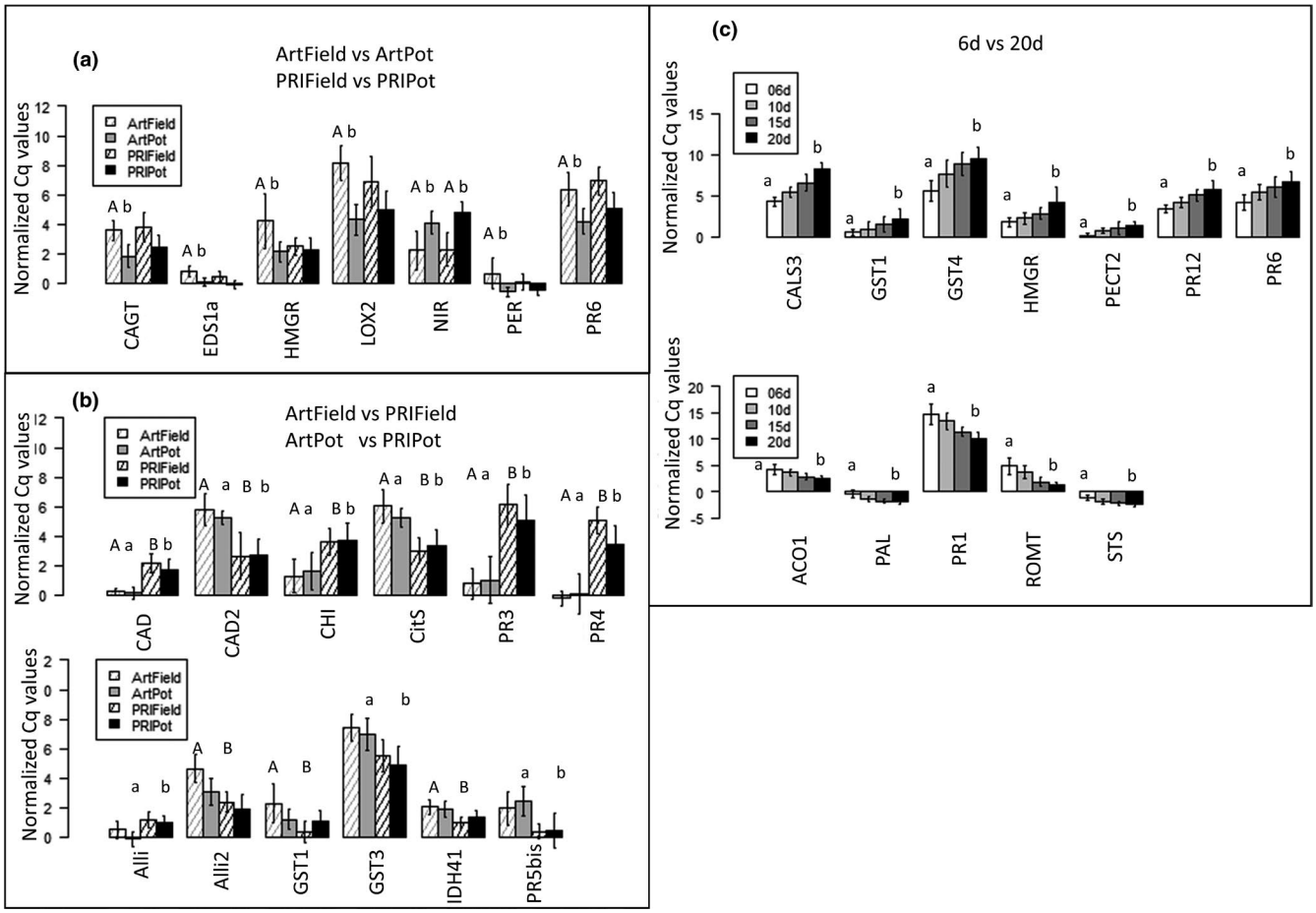

FIGURE 3 Effect of growing conditions (pot vs. field) on gene expression of *Vv* genes on resistant grapevine varieties Artaban (Art) and Prior (PRI). (a) Effect of growing conditions on the same variety; (b) effect of variety on the same growing condition; (c) effect of leaf age (6 to 20 days) regardless of the variety or condition. Only bars with significant differences according to the multiple *t* test are indicated by different letters (limmaCtData function from R package HTqPCR). Bars with letters in upper case indicate significant differences between field data, and lower case letters indicate significant differences between pot data or the most extreme leaf age

genes increases, the disease decreases (negative correlation–black line on Figure 9): 'PR Proteins', 'Ethylene-SA', and 'Phenylpropanoids', with relative contributions to 'Disease' of 25.4%, 15.3%, and 11.3%, respectively. Three metabolic pathways were positively correlated with the 'Disease' (positive correlation– grey line): 'Cell wall', 'Primary metabolism', and 'Signalling', with relative contributions to 'Disease' of 17.7%, 18.3%, and 11.9%, respectively. Thus, when the expression of selected genes in these pathways increases, 'Disease' increases. Furthermore, the analysis revealed that 77% of PR protein pathway variation was explained by both 'Leaf age' (69.9% relative contribution) and 'Varieties' (CR2 = 30.1%), whereas 'Phenylpropanoids' and 'Ethylene-SA' pathway variations were mainly explained by 'Leaf age' (96.5% and 77.5%, respectively). 'Cell wall' was also mainly explained by 'Leaf age' variation (82.1%), whereas 'Primary metabolism' and 'Signalling' were mainly explained by 'Varieties' (64.8% and 55.7%, respectively); cross-loadings (correlation between manifest variables and latent variables) provided some clues about the variables with the main effect on the three disease variables: spore germination was mostly negatively correlated to 'PR proteins' (−0.79) (mainly through *VvPR3*, *VvPR4*, and *VvPR15*), 'Ethylene-SA' (−0.67) (*VvACO1*) and 'Phenylpropanoids' (−0.62) (*VvCHS*, *VvLDOX*, *VvDFR*), while it was increased for high levels of *VvPER* from 'Cell wall reinforcement' (0.69); spore ramification was negatively correlated to 'PR proteins' (−0.71) and increased when genes from 'Primary metabolism' were highly expressed (0.69) (*VvNADH*); and sporulation was mainly negatively correlated to 'PR proteins' (−0.66) and positively correlated to 'Primary metabolism' (0.65) and 'Signalisation' (0.61). The global 'Disease' variable was negatively correlated with *VvACO1* (−0.66), *VvPR4* (−0.62), *VvPR3* (−0.60), *VvLDOX* (−0.57), and *VvDFR* (−0.56), to variety Artaban (−0.61), and to the 20 days leaf age (−0.52), and it was positively correlated with *VvPER* (0.72), *VvWRKY2* (0.54), *VvCitS* (0.54), and *VvNADH* (0.59), and to the 6 days leaf age (0.56) (Table S2). The relative goodness of fit of the model was 0.90. If instead of the 'Leaf age' latent variable, a 'Physiology' variable was used with continuous manifest of Chl, Flav, 1/Ant, and LS (leaf surface), almost the same results were obtained because data for metabolic pathways were identical, but the correlations were slightly improved between metabolic pathways and 'Physiology'. According to the cross-loadings, spore germination was mostly negatively correlated with physiological characteristics (−0.82) compared to sporulation (−0.65).

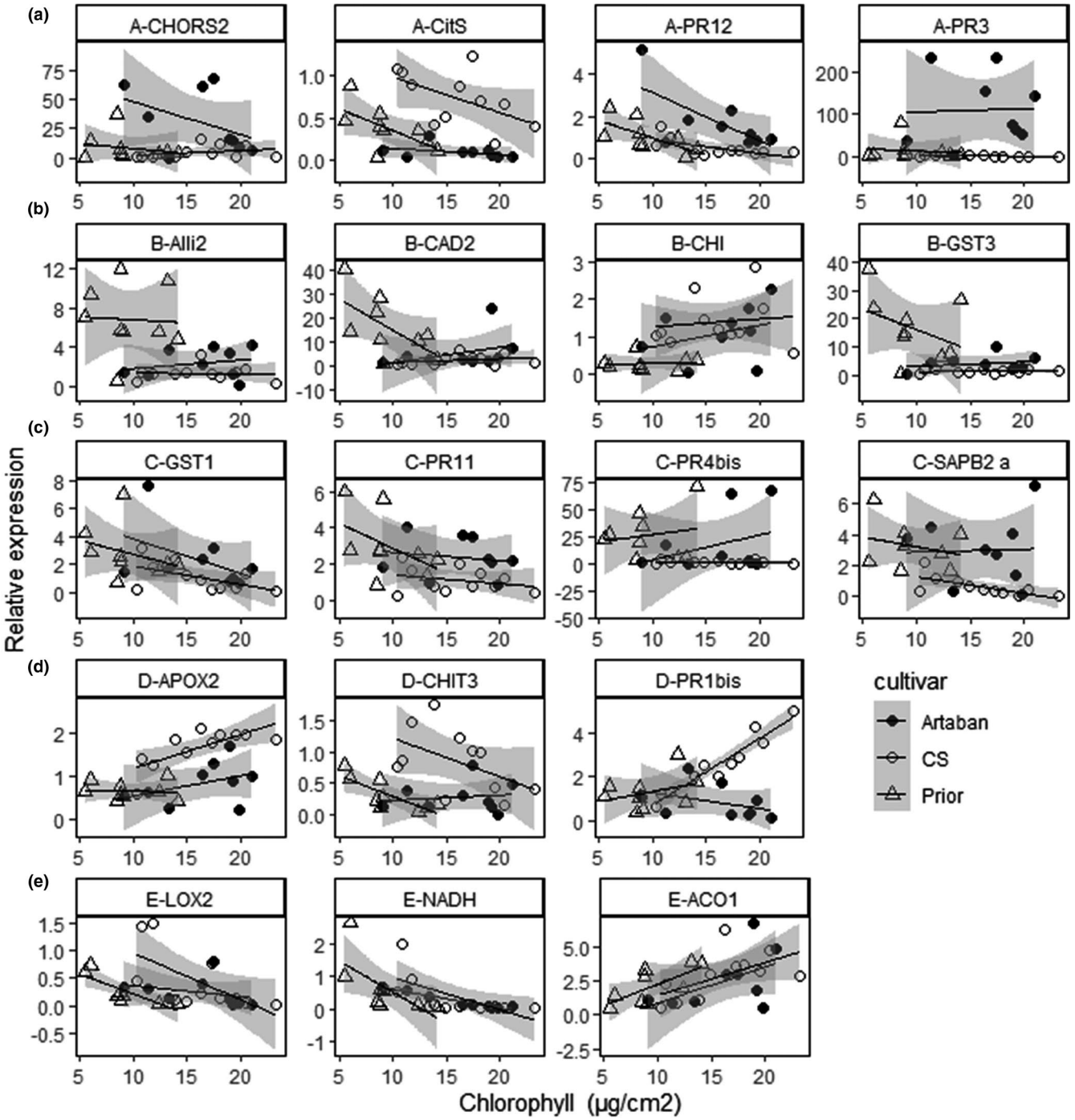

FIGURE 4 Relative expression as a function of leaf chlorophyll content for discriminant genes before inoculation with powdery mildew. Leaves were sampled from the three cultivars Artaban, Cabernet Sauvignon (CS), and Prior from 2-year-old grafted grapevines. Genes are grouped in a, b, c, d, e for markers of Artaban (a), Prior (b), both resistant varieties (c), Cabernet Sauvignon (d), or function of leaf age (e). The grey band is the confidence band for the regression line. For gene classification and names, see Table S1

4 | DISCUSSION

Our study investigated the gene expressions and pathogenicity responses to *E. necator* by susceptible and resistant grapevine varieties. We used the same leaves for measurements to take into account the ontogenic development of the plant and to measure its effect on pathogenicity traits as well as on defence. We observed a significant decrease in germination and sporulation regardless of leaf age for the three varieties. The experimental design and the analyses used made it possible to determine correlations between the plant's features and disease variables and to discriminate variety-specific defences, whether constitutive or induced by powdery mildew, from basal defences related to ontogenic resistance.

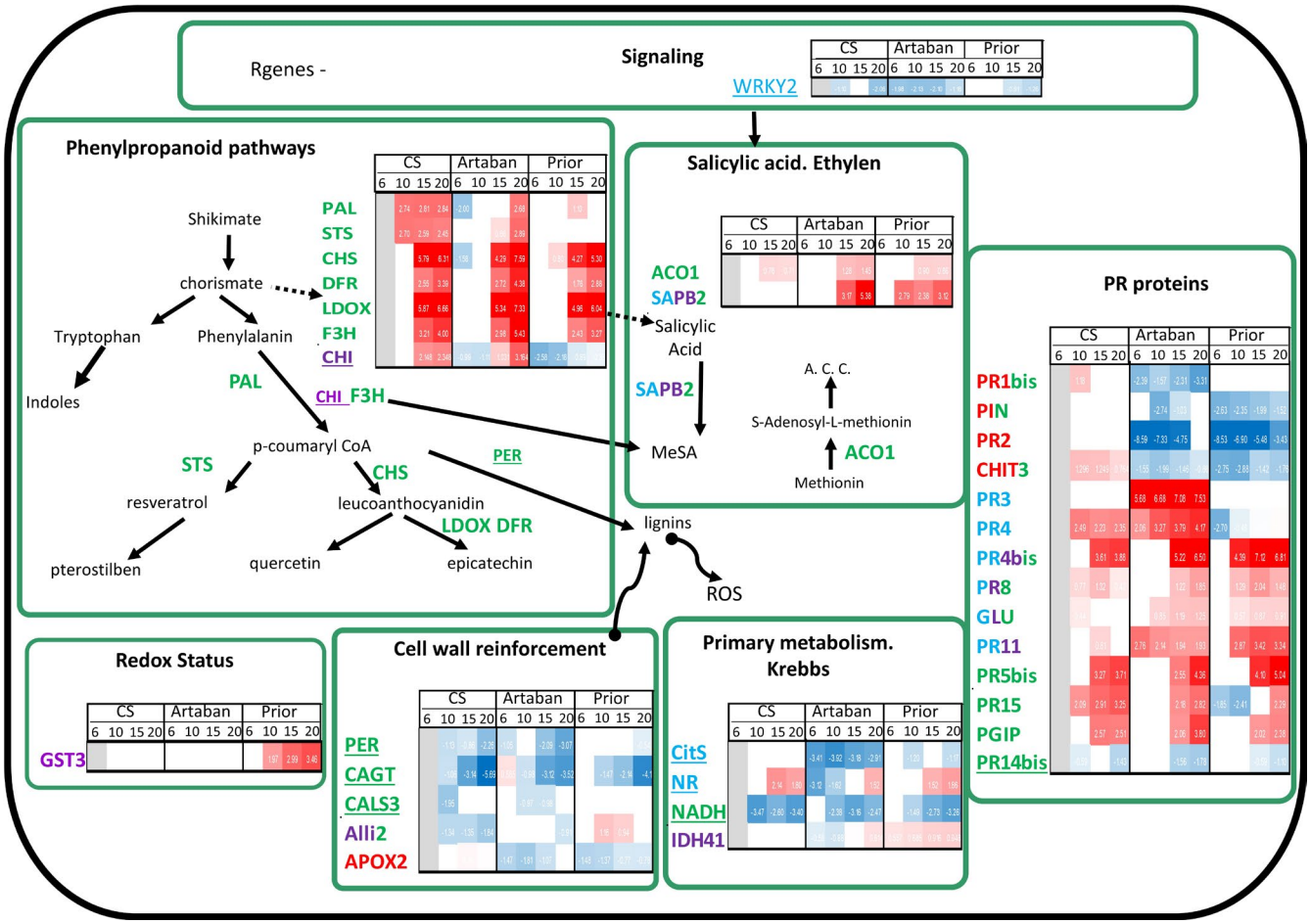

FIGURE 5 Summary diagram representing the genes of the different metabolic pathways that are overexpressed (bold) or repressed (underlined) for each of the three varieties, Cabernet Sauvignon (CS) (red), Artaban (blue), and Prior (purple), after inoculation with powdery mildew, or the genes overexpressed vs repressed according to leaf age (green). Double colours indicate differential expression for a variety according to the age of the leaf. For each gene, variety and leaf age (d6, d10, d15, d20) a pattern of the relative expression at 24 hr postinoculation (hpi) is indicated. Up-regulated genes appear in shades of red, with an expression level higher than 5 shown in bright red, while those that were down-regulated appear in shades of blue, with an intensity lower than −5 shown in dark blue. Relative expression data are averaged (three or four repetitions) according to the leaf age and $\log_2$ transformed. Gene expression of CS-6d inoculated leaves was used as a reference to calculate the relative expression. Numbers in boxes represent significant changes in gene expression ($p \leq 0.05$) compared with the reference. White colour indicates no significant difference from the reference according to Dunnett's test. Arrows indicate the relationships between metabolic pathways

For Artaban, all phases of the powdery mildew life cycle were affected, with a decrease in germination, mycelial growth, and a total inhibition of sporulation. The absence of sporulation was most likely the consequence of reduced mycelial growth subsequently resulting in the lack of formation of haustoria. Differences between CS and Prior were weak, especially for sporulation, and the early phases of PM development (germination and mycelial growth) even appeared to be favoured on the hybrid Prior when compared with the *V. vinifera* susceptible variety CS, with no sign of incompatible interaction. A significant effect of leaf age was observed on germination and mycelial growth for the three varieties.

Differences in gene expression were based on a limited number of genes but they were such that varieties could be distinguished by a hierarchical clustering analysis. The contribution of certain PR proteins in defence as well as some regulatory mechanisms involving genes from primary metabolism, development, or transcription factors was highlighted for each variety. The experiments and statistical methods used allowed us to quantify the contribution to the disease of genotypic expression involved in genetic resistance (variety) and in ontogenic resistance (leaf age) and then allowed us to quantify the contribution of each metabolic pathway to the disease.

After inoculation, most of the PR protein genes were induced either at very high levels in resistant varieties for *VvPR3* and *VvPR4* (relative expression up to 200 and 20, respectively) or *VvPR4bis* (relative expression up to 150) or at moderate increasing levels with leaf age for *VvGLU*, *VvPR8*, or *VvPR11*, mostly for the oldest leaves. In the highly resistant variety Artaban, the *VvPR3* gene was constitutively overexpressed in all leaves, regardless of the growth condition. Its expression was strongly enhanced after inoculation with PM and increased with leaf age, whereas in CS and Prior, its expression

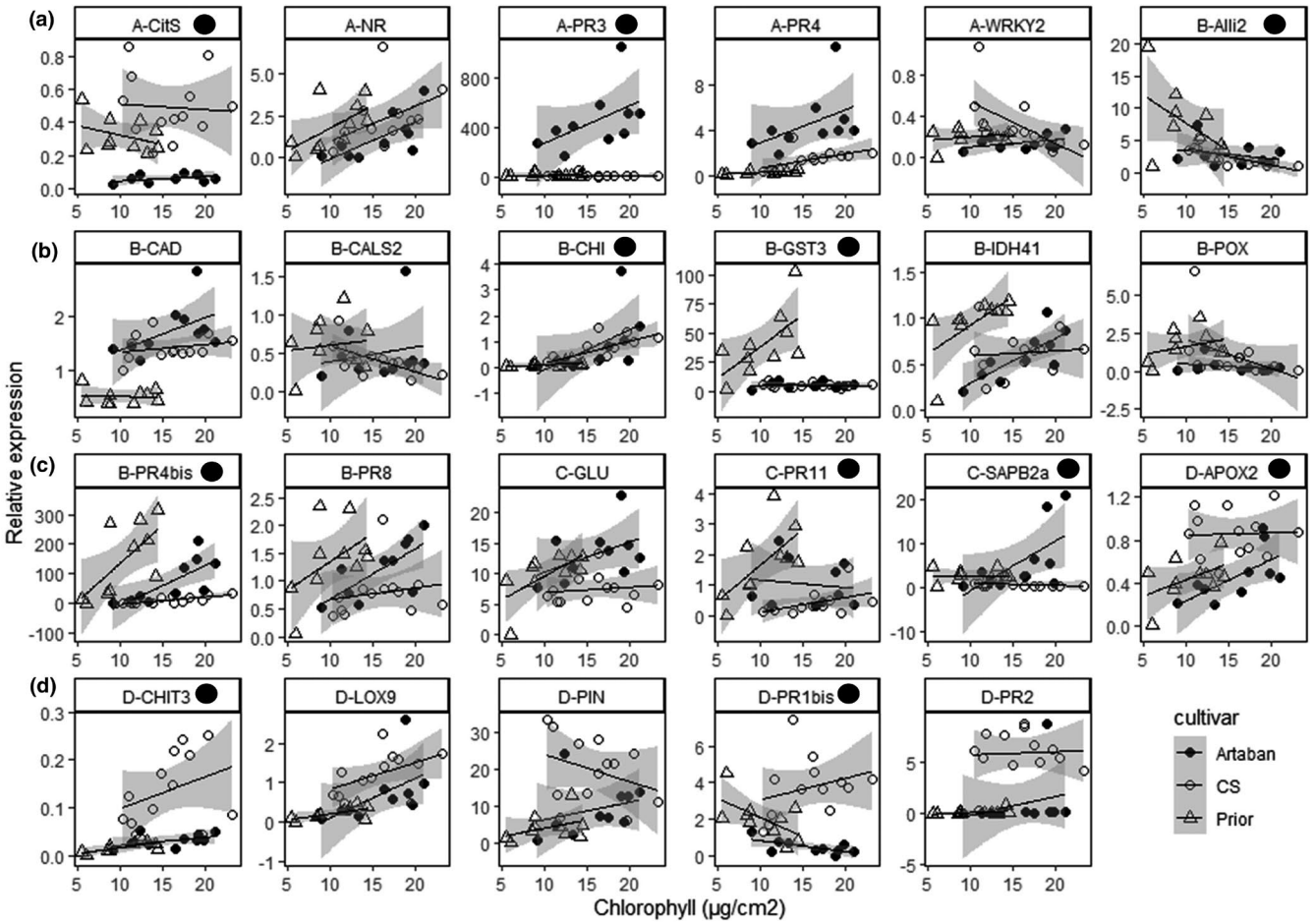

FIGURE 6 Relative expression as a function of leaf chlorophyll content for discriminant genes 24 hr after inoculation. Leaves were sampled from the three cultivars Artaban, Cabernet Sauvignon (CS), and Prior from 2-year-old grafted grapevines. Genes are grouped in a, b, c, and D-Vvgene name for markers of Artaban (a), Prior (b), both resistant varieties (c), and Cabernet Sauvignon (d), respectively. The grey band is the confidence band for the regression line. Black dots indicate genes that were already discriminatory before inoculation. For gene classification and gene names, see Table S1

increased only after inoculation and to a much lower level that in Artaban. This PR3 protein is an endochitinase class IV. Among PR3s, in general, class I endochitinases have the highest antifungal activity, although there are some examples in the literature of antifungal activity for endochitinase IV in yam, papaya, and *Arabidopsis*. In grapevine, the comparison of gene expression by the Affymetrix method highlighted the overexpression of a type IV endochitinase in *V. aestivalis* (cv. Norton) compared to *V. vinifera* (cv. CS) (Fung et al., 2007). In *V. vinifera*, type IV endochitinase can be expressed at specific developmental stages, that is, maturing grape berries or flowers (Colas et al., 2012), and can be induced in leaves when the plant is elicited with benzothiadiazole (Dufour et al., 2013). A class IV chitinase from yam was even tested as a biocontrol agent after spraying on strawberry infected by PM with an effect enhanced by the addition of β-1,3-glucanase (Karasuda et al., 2003). A correlation between a combined constitutive level of chitinase and β-1,3-glucanase activities in various grapevine cultivars and their field resistance to PM infection supported the hypothesis that tissues containing constitutively expressed PR genes were more resistant to pathogen attack (Giannakis et al., 1998). The levels of both enzymes were generally found in lower amounts in the *V. vinifera* cultivars than in hybrids or other *Vitis* (especially *V. rupestris*, *V. labrusca*, and Seyval). The very high and constitutive expression of *VvPR3* and *VvPR4* in Artaban was most likely responsible for the significant decline in spore germination and mycelial growth inhibition. Susceptible grapevine cultivars inoculated with PM showed markedly increased activity levels of a chitinase and a β-1,3-glucanase in the leaves (Jacobs et al., 1999), and this was associated with the gene expression of *VvPR2*, *VvPR3*, and *VvPR5* families. A significant increase in the expression of the β-1,3-glucanase gene *VvGLU* was also observed in our results after inoculation for all leaves of the three varieties and at higher levels for the oldest leaves of resistant varieties. A synergistic effect between β-1,3-glucanase and chitinases (PR3 and PR4 for Artaban, PR4bis for Prior) may increase the global level of resistance of the oldest leaves of the resistant varieties. However, our results indicated that not all chitinases and β-1,3-glucanases were efficient against PM, because in CS, *VvCHIT3a* and *VvPR2* had apparently no effect on the disease, or they were expressed at a level too low to have an effect. The other genes coding for PR proteins (*VvPR15*, *VvPR5bis*, *VvPGIP*, and *VvCHIT4a*) for which expression increased with leaf

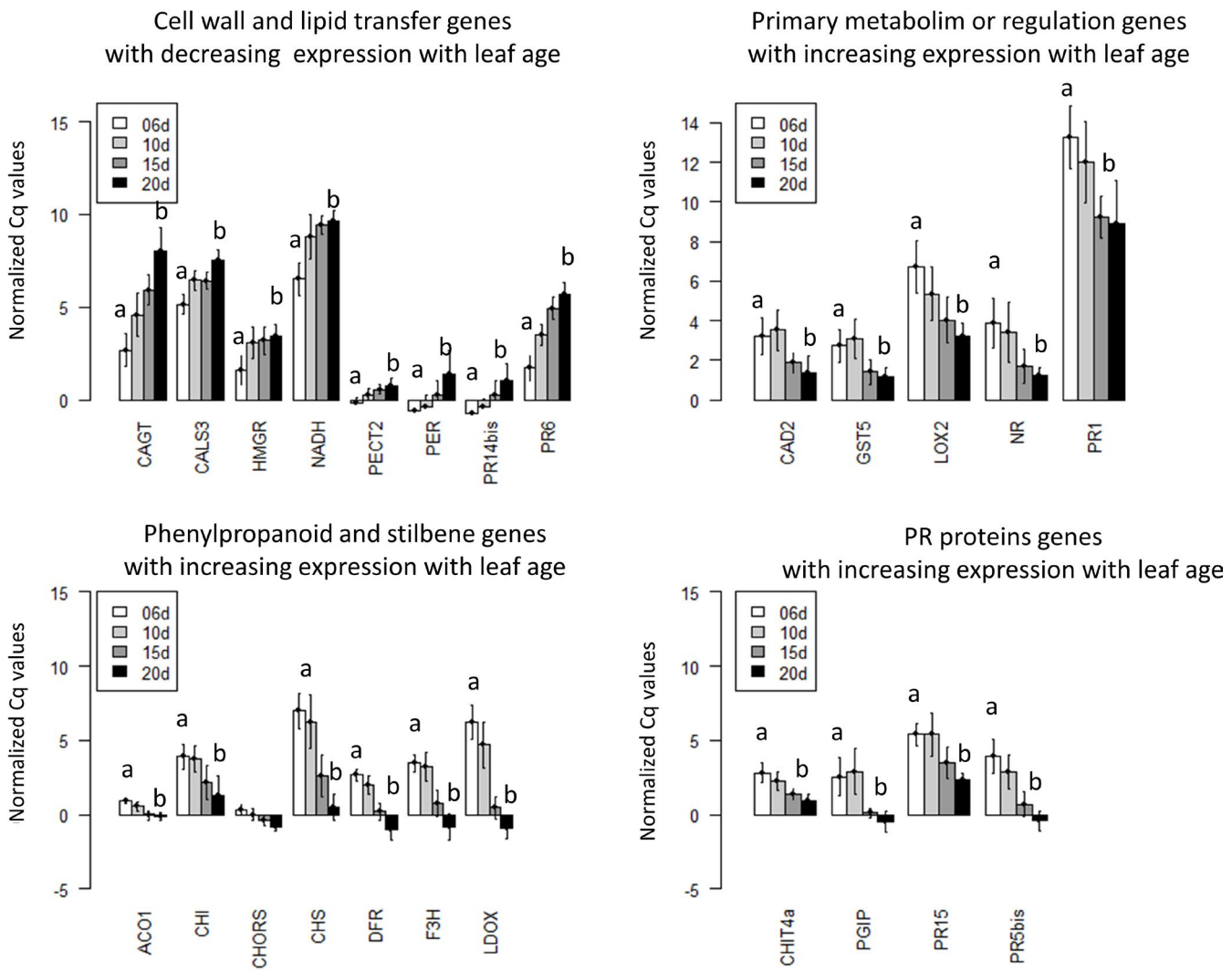

FIGURE 7 Differences in expression of selected genes as affected by leaf age of potted vines, after inoculation with powdery mildew. Each gene is represented by 9 to 11 samples (per leaf age). Bars with different letters are significantly different according to multiple *t* test (limmaCtData function from R package HTqPCR). For gene classification and names, see Table S1

age for the three varieties might contribute to ontogenic resistance. The expression of germin-like protein-*VvPR15* and thaumatin-like protein-*VvPR5bis* was correlated with leaf age and the cell wall and ethylene–SA pathways, respectively. Polygalacturonase-inhibiting protein (PGIP), found in the cell wall of many plants, counteracted the cell wall-degrading enzymes produced by the pathogen by forming specific complexes with them. Only three PR proteins decreased with leaf age: PR14bis, a lipid transfer protein, and the two serine proteases PR6 and PIN in CS. Altogether, the gene expression of the main PR proteins was more correlated with the germination (−0.79) and ramification of spores (−0.71) and at least accounted for 25% of disease variation.

The phenylpropanoid pathway yield two major flavonoid and stilbene groups that are involved as grapevine phytoalexins. Our results showed that genes from the ethylene and phenylpropanoid pathways were overexpressed in the oldest leaves without differential expression between the three varieties. In the phenylpropanoid pathway, *VvPAL*, *VvSTS*, *VvROMT*, and *VvACC* were constitutively overexpressed before inoculation in the oldest leaves. However, these genes were repressed by the pathogens after inoculation and only those of the older leaves were highly expressed compared to the youngest inoculated leaves of CS. It has previously been shown that *VvSTS* genes were more induced by PM in CS than in *V. aestivalis* 'Norton' (Dai et al., 2012), with lower expression levels in young than in old leaves, similar to that observed in our study. *VvACO1* was overexpressed with leaf development but was also overexpressed in all leaves after inoculation, which is consistent with regulation of the response to attack by PM. However, the role of ACC oxidase in defence may be nuanced. ACC oxidase has been shown to comprise multigene families in many species, some displaying tissue-specific and developmentally regulated expression (Chen & McManus, 2006). The expression of several genes involved in the phenylpropanoid pathway such as *VvCHS*, *VvDFR*, *VvLDOX*, and *VvF3H* was highly correlated with the global PR protein and the ethylene-SA variables (cross loading >0.75). These genes clearly participate in basal defence and may reflect the basic plant-triggered immunity of

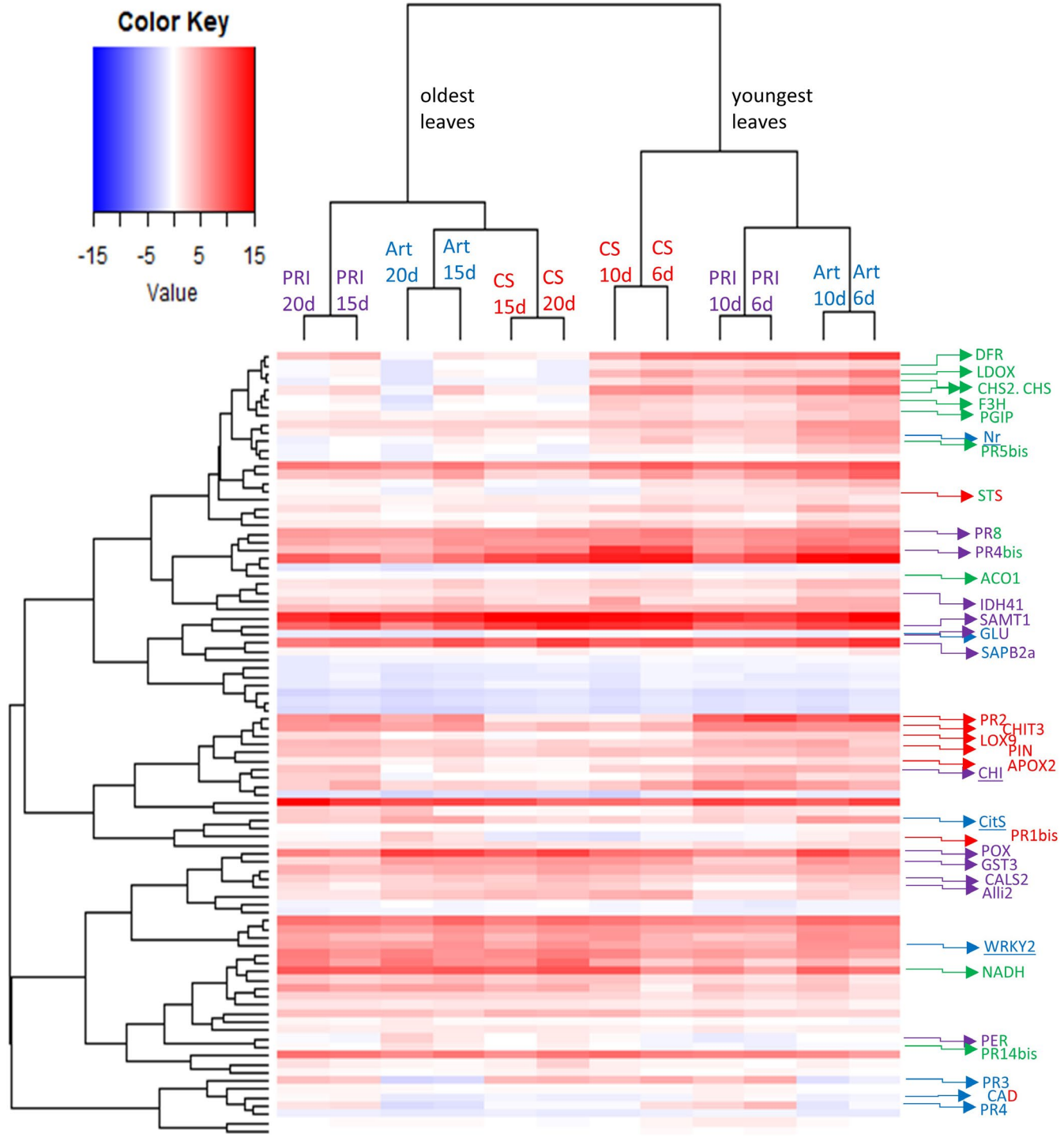

FIGURE 8 Dendogram resulting from the hierarchical clustering analysis based on Pearson correlation distance, grouping samples of resistant varieties × leaf age with the closest C_q values for the whole set of features 24 hr after inoculation with powdery mildew using potted grapevines. Each individual data is represented by four to six samples per leaf age (6, 10, 15, 20 days) per variety. The genes that discriminate the Artaban (Art) variety are indicated in blue, those for Prior (PRI) are indicated in purple, those for Cabernet Sauvignon (CS) are indicated in red, and those that vary with leaf age are indicated in green

grapevines. Their expression is mainly correlated with spore germination (−0.62) and to the oldest leaves (−0.67). One gene of the salicylic acid (SA) pathway, *VvSAPB2*, was also slightly and significantly overexpressed in both resistant varieties, especially for the oldest leaves in Artaban.

In association with the overexpression of PR proteins, the variety Artaban showed a repression of the *VvCitS* gene, an enzyme involved in the Krebs cycle, as well as lower expression of nitrate reductase. Plant defence is energy intensive, and constitutive PR gene expression could result in modification of primary growth (Bolton, 2009).

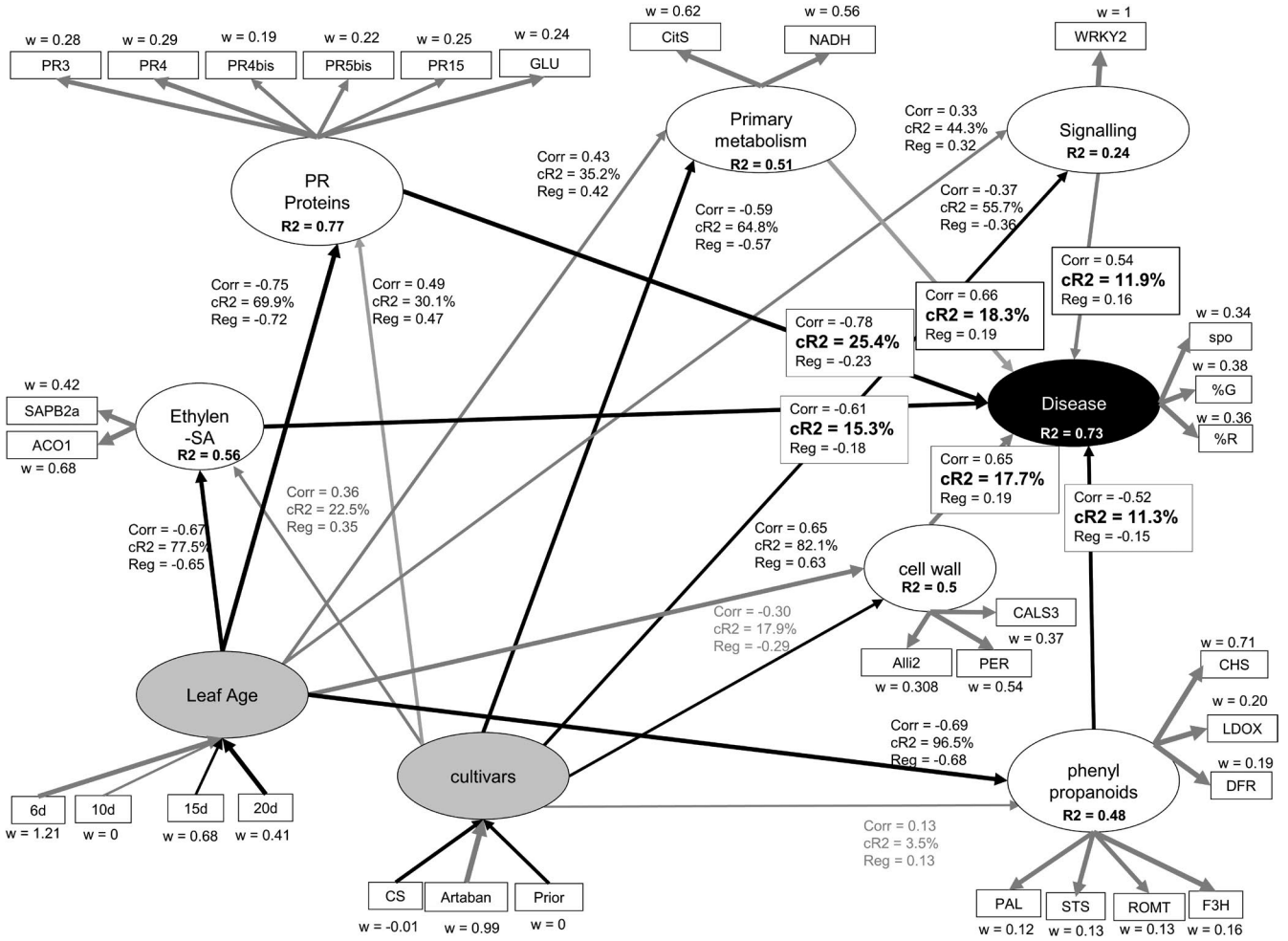

FIGURE 9 Output of the partial least squares path modelling describing the relationships between the endogenous latent variable 'Disease' and the other latent exogenous variables 'PR proteins', 'Ethylene-SA', 'Primary metabolism', 'Signalling', 'Cell wall', 'Phenylpropanoids', 'Leaf age', and 'Cultivars'. Corr indicates the correlation coefficient between two latent variables, CR2 indicates the relative contribution of exogenous latent variables to the endogenous one, Reg indicates the value of the path coefficient, R^2 indicates the regression coefficient between two latent variables, and w indicates the outer weights yielding the covariances between the manifest variable and its inner latent variable. Grey lines indicate positive correlations, and black lines indicate negative correlations. Values in grey indicate no significant correlations

However, a plant can manage regulation of the Krebs cycle without disastrous consequences (Sienkiewicz-Porzucek et al., 2008), and levels of chlorophyll content and NBI of Artaban leaves were within standards. Our physiological measurements showed that compared to CS, both resistant varieties invested more in protecting compounds such as high contents of chlorophyll and flavonols and low content of anthocyanins for Artaban and low content of chlorophyll and high contents of anthocyanins and flavonols for Prior. However, the very early reddening of Artaban leaves in the field suggests physiological deregulation. In contrast, for Prior, the overexpression of *VvIDH41* and *VvGST1* in the field was an indication of high Krebs cycle activity for the variety, presumably in relation to its vigorous growth. Another marker for Prior, *VvGST3*, was overexpressed before and mostly after inoculation, with increased expression for the oldest leaves. This may also be a component of ontogenic resistance by playing a role in detoxification of toxic substances, attenuation of oxidative stress, participation in hormone transport, or as a receptor protein for SA. Functional studies revealed that overexpression of specific glutathione S-transferases can modify disease symptoms and pathogen multiplication rates (Soustre-Gacougnolle et al., 2018).

Regarding the cell wall, strong expression of *VvCAD2* in the absence of inoculation in the oldest leaves of resistant varieties, particularly in Prior, suggests a possible reinforcement of the synthesis of monolignols and lignin in leaves that could play a role in resistance, as described for resistant wheat varieties (Ma, 2010). The other genes known to be involved in membrane strengthening (*VvPER*, *VvCALS3*, *VvPECT2*, *VvPOX*, *VvAlli2*) showed a decrease in expression with the age of the leaves after inoculation, which was consistent with a reaction to pathogen attack and their pivotal role in cell wall loosening in the presence of H_2O_2 (Francoz et al., 2015).

Transcription factors were either overexpressed for *VvWRKY1* in all leaves from the three varieties after inoculation or repressed for *VvWRKY2* in Artaban and the oldest leaves of Prior and CS. The overexpression of *VvWRKY2* in CS was positively correlated with

the disease. This supports the hypothesis of detection of the PAMP by WRKY1 and of the interaction between WRKY2 and the protein coded by the resistance gene for Artaban. In barley, WRKY1 and WRKY2 act as repressors of PAMP-triggered basal defence against powdery mildew. WRKY2 combines with an MLA protein involved in resistance, which derepresses basal defence. When *VvWRKY2* is overexpressed, the kinetics of the interaction with MLA are inadequate and compromise the immune response (Shen et al., 2007).

It is important to note that factors of variation were identified in this work. The high susceptibility of Prior in our bioassay was in contrast to its high level of field resistance under natural inoculation. Its field resistance might be derived from its notable early bud burst and its particularly rapid growth, with an effect of the time lag between the amount of receptive leaf tissue and the onset of epidemics. The variety markers identified with overexpression of *VvCAD2* involved in lignin synthesis in the oldest leaves before inoculation and of *VvPR4bis*, *VvPR8*, *VvPR11*, *VvAlli2*, and *VvGST3* after inoculation observed in our study might enhance its ontogenic resistance in the field, especially in leaves subjected to other pests, wounds, and UV-B light. The very high constitutive overexpression of *VvPR3* in Artaban and its particular modulation of primary metabolism was unusual and has never been cited in the literature as associated with genotypes carrying *Run1*, even if overexpression of a *VvPR3* is associated with complete resistance to *V. aestivalis*. When we compared the gene expression for potted and field plants, differences in gene expression were observed for a few genes related to primary growth and strongly influenced by the stage of development (*VvNiR*, *VvPER*, *VvLOX2*, *VvCAGT*, *VvPR6*, *VvHMGR*). It is therefore likely that the variety marker genes we have identified persist in varying weather conditions and plant growth patterns but variation of environmental conditions could modify their expression or induce other genes involved in defence. The next step will be to compare closely and distantly related genotypes to better understand the effect of the genetic background and environmental conditions on the plant's defence mechanisms with respect to different pathogens and for different organs such as bunches.

To our knowledge, this study represents the first investigation on genotypic expression involved in genetic (variety) and ontogenic (leaf age) resistances of different grapevine varieties to powdery mildew. We hope this information will provide knowledge for vine management based on gene expression, and potentially make better use of stimulators of plant defence and/or optimize cultivation practices to obtain the most efficient and least vulnerable plants (Soustre-Gacougnolle et al., 2018). It will further define grapevine ideotypes: genotypes adapted to environmental conditions, including ontogeny-specific knowledge that can be cultivated in a more sustainable way in line with disease pressures.

ACKNOWLEDGEMENTS

The authors gratefully thank Philippe Cartolaro, Gilles Taris, Lionel Druelle, and Sébastien Gambier from INRAe UMR 1065 SAVE for the maintenance of the experimental plants and the staff of the INRAe experimental unit for planting the ANTARES plot in 2016. We would also like to thank P. Coutos-Thevenot from Univ. of Poitier for the NADH-plastoquinone oxidoreductase gene primers, and P. Bloy and L. Audeguin from the French Institute of Vine and Wine (IFV) for providing the Artaban plants. The manuscript benefited from the editing efforts of Elsevier editing service and Denis Thiéry INRAe UMR 1065 SAVE. Research reported in this publication was supported by French Biodiversity Agency (OFB) as part of the call of a CASDAR project launched by the French Ministries in charge of Ecology (MTES), Agriculture (MAA), Health (MSS) and Research (MESRI).

CONFLICT OF INTEREST

The authors declare that this research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

DATA AVAILABILITY STATEMENT

The data sets generated during and/or analysed for the current study are available from the corresponding author upon request.

ORCID

Agnes Calonnec https://orcid.org/0000-0001-6027-6138

REFERENCES

Bois, B., Zito, S. & Calonnec, A. (2017) Climate vs grapevine pests and diseases worldwide: the first results of a global survey. *OENO One*, *51*, 133–139.

Bolton, M.D. (2009) Primary metabolism and plant defense—fuel for the fire. *Molecular Plant-Microbe Interactions*, *22*, 487–497.

Brulé, D., Villano, C., Davies, L.J., Trdá, L., Claverie, J., Héloir, M.C., Chiltz, A. et al (2018) The grapevine (*Vitis vinifera*) LysM receptor kinases VvLYK1-1 and VvLYK1-2 mediate chitooligosaccharide-triggered immunity. *Plant Biotechnology Journal*, *17*, 812–825.

Cadle-Davidson, L., Mahanil, S., Gadoury, D.M., Kozma, P. & Reisch, B.I. (2011) Natural infection of *Run1*-positive vines by naive genotypes of *Erysiphe necator*. *Vitis*, *50*, 173–175.

Calonnec, A., Jolivet, J., Vivin, P. & Schnee, S. (2018) Pathogenicity traits correlate with the susceptible *Vitis vinifera* leaf physiology transition in the biotroph fungus *Erysiphe necator*: an adaptation to plant ontogenic resistance. *Frontiers in Plant Science*, *9*, 1808.

Calonnec, A., Wiedemann-Merdinoglu, S., Delière, L., Cartolaro, P., Schneider, C. & Delmotte, F. (2013) How reliable are leaf bioassays for predicting disease resistance on fruit: a case study on grapevine resistance to downy and powdery mildew. *Plant Pathology*, *62*, 533–544.

Cerovic, Z.G., Masdoumier, G., Ghozlen, N.B. & Latouche, G. (2012) A new optical leaf-clip meter for simultaneous non-destructive assessment of leaf chlorophyll and epidermal flavonoids. *Physiologia Plantarum*, *146*, 251–260.

Chen, B.C.M. & McManus, M. (2006) Expression of 1-aminocyclopropane-1-carboxylate (ACC) oxidase genes during the development of vegetative tissues in white clover (*Trifolium repens* L.) is regulated by ontological cues. *Plant Molecular Biology*, *60*, 451–467.

Colas, S., Afoufa-Bastien, D., Jacquens, L., Clément, C., Baillieul, F., Mazeyrat-Gourbeyre, F. et al (2012) Expression and *in situ* localization of two major PR proteins of grapevine berries during development and after UV-C exposition. *PLoS One*, *7*, e43681.

Dai, R., Ge, H., Howard, S. & Qiu, W. (2012) Transcriptional expression of stilbene synthase genes are regulated developmentally

and differentially in response to powdery mildew in Norton and Cabernet Sauvignon grapevine. *Plant Science*, *197*, 70–76.

Dry, I.B., Feechan, A., Anderson, C., Jermakow, A.M., Bouquet, A., Adam-Blondon, A.-F. et al (2010) Molecular strategies to enhance the genetic resistance of grapevines to powdery mildew. *Australian Journal of Grape and Wine Research*, *16*, 94–105.

Dufour, M.-C., Lambert, C., Bouscaut, J., Mérillon, J. & Corio-Costet, M.-F. (2013) Benzothiadiazole-primed defense responses and enhanced differential expression of defense genes in *Vitis vinifera* infected with biotrophic pathogens (*Erysiphe necator* and *Plasmopara viticola*). *Plant Pathology*, *62*, 370–382.

Dufour, M.-C., Magnin, N., Dumas, B., Vergnes, S. & Corio-Costet, M.-F. (2016) High-throughput gene-expression quantification of grapevine defense responses in the field using microfluidic dynamic arrays. *BMC Genomics*, *17*, 957.

Dvinge, H. & Bertone, P. (2009) HTqPCR: High-throughput analysis and visualization of quantitative real-time PCR data in R. *Bioinformatics*, *25*, 3325.

Feechan, A., Anderson, C., Torregrosa, L., Jermakow, A., Mestre, P., Wiedemann-Merdinoglu, S. et al (2013) Genetic dissection of a TIR-NB-LRR locus from the wild North American grapevine species *Muscadinia rotundifolia* identifies paralogous genes conferring resistance to major fungal and oomycete pathogens in cultivated grapevine. *The Plant Journal*, *76*, 661–674.

Francoz, E., Ranocha, P., Nguyen-Kim, H., Jamet, E., Burlat, V. & Dunand, C. (2015) Roles of cell wall peroxidases in plant development. *Phytochemistry*, *112*, 15–21.

Fung, R.W.M., Gonzalo, M., Fekete, C., Kovacs, L.G., He, Y., Marsh, E. et al (2008) Powdery mildew induces defense-oriented reprogramming of the transcriptome in a susceptible but not in a resistant grapevine. *Plant Physiology*, *146*, 236–249.

Fung, R.W.M., Qiu, W., Su, Y., Schachtman, D.P., Huppert, K., Fekete, C. et al (2007) Gene expression variation in grapevine species *Vitis vinifera* L. and *Vitis aestivalis* Michx. *Genetic Resources and Crop Evolution*, *54*, 1541–1553.

Gamir, J., Darwiche, R., van't Hof, P., Choudhary, V., Stumpe, M., Schneiter, R. et al (2017) The sterol-binding activity of PATHOGENESIS-RELATED PROTEIN 1 reveals the mode of action of an antimicrobial protein. *The Plant Journal*, *89*, 502–509.

Giannakis, C., Bucheli, C.S., Skene, K.G.M., Robinson, S.P. & Scott Steele, N. (1998) Chitinase and β-1,3-glucanase in grapevine leaves: a possible defence against powdery mildew infection. *Australian Journal of Grape and Wine Research*, *4*, 14–22.

Hayes, M.A., Feechan, A. & Dry, I.B. (2010) Involvement of abscisic acid in the coordinated regulation of a stress-inducible hexose transporter (VvHT5) and a cell wall invertase in grapevine in response to biotrophic fungal infection. *Plant Physiology*, *153*, 211–221.

Hellemans, J., Mortier, G., De Paepe, A., Speleman, F. & Vandesompele, J. (2007) qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. *Genome Biology*, *8*, R19.

Jacobs, A.K., Dry, I. & Robinson, S.P. (1999) Induction of different pathogenesis-related cDNAs in grapevine infected with powdery mildew and treated with ethephon. *Plant Pathology*, *48*, 325–336.

Karasuda, S., Tanaka, S., Kajihara, H., Yamamoto, Y. & Koga, D. (2003) Plant chitinase as a possible biocontrol agent for use instead of chemical fungicides. *Bioscience, Biotechnology and Biochemistry*, *67*, 221–224.

Leinhos, G.M.E., Gold, R.E., Duggelin, M. & Guggenheim, R. (1997) Development and morphology of *Uncinula necator* following treatment with the fungicides kresoxim-methyl and penconazole. *Mycological Research*, *101*, 1033–1046.

Ma, Q.-H. (2010) Functional analysis of a cinnamyl alcohol dehydrogenase involved in lignin biosynthesis in wheat. *Journal of Experimental Botany*, *61*, 2735–2744.

Mammeri, Y., Burie, J.B., Langlais, M. & Calonnec, A. (2014) How changes in the dynamic of crop susceptibility and cultural practices can be used to better control the spread of a fungal pathogen at the plot scale? *Ecological Modelling*, *290*, 178–191.

Meng, X. & Zhang, S. (2013) MAPK Cascades in plant disease resistance signaling. *Annual Review of Phytopathology*, *51*, 245–266.

Merdinoglu, D., Schneider, C., Prado, E., Wiedemann-Merdinoglu, S. & Mestre, P. (2018) Breeding for durable resistance to downy and powdery mildew in grapevine. *OENO One*, *52*, 203–209.

Meyer, S., Cerovic, Z.G., Goulas, Y., Montpied, P., Demotes-mainard, S., Bidel, L.P.R. et al (2006) Relationships between optically assessed polyphenols and chlorophyll contents, and leaf mass per area ratio in woody plants: a signature of the carbon-nitrogen balance within leaves? *Plant, Cell and Environment*, *29*, 1338–1348.

Montaigne, E., Coelho, A. & Khefifi, L. (2016) Economic issues and perspectives on innovation in new resistant grapevine varieties in France. *Wine Economics and Policy*, *5*, 73–77.

Pauquet, J., Bouquet, A., This, P. & Adam-Blondon, A.F. (2001) Establishment of a local map of AFLP markers around the powdery mildew resistance gene *Run1* in grapevine and assessment of their usefulness for marker assisted selection. *Theoretical and Applied Genetics*, *103*, 1201–1210.

Peterson, C., Eviner, V. & Gaudin, A. (2018) Ways forward for resilience research in agroecosystems. *Agricultural Systems*, *162*, 19–27.

Qiu, W.P., Feechan, A. & Dry, I. (2015) Current understanding of grapevine defense mechanisms against the biotrophic fungus (*Erysiphe necator*), the causal agent of powdery mildew disease. *Horticulture Research*, *2*, 15020.

Shen, Q.-H., Saijo, Y., Mauch, S., Biskup, C., Bieri, S., Keller, B. et al (2007) Nuclear activity of MLA immune receptors links isolate-specific and basal disease-resistance responses. *Science*, *315*, 1098–1103.

Sienkiewicz-Porzucek, A., Nunes-Nesi, A., Sulpice, R., Lisec, J., Centeno, D.C., Carillo, P. et al (2008) Mild reductions in mitochondrial citrate synthase activity result in a compromised nitrate assimilation and reduced leaf pigmentation but have no effect on photosynthetic performance or growth. *Plant Physiology*, *147*, 115–127.

Soustre-Gacougnolle, I., Lollier, M., Schmitt, C., Perrin, M., Buvens, E., Lallemand, J.-F. et al (2018) Responses to climatic and pathogen threats differ in biodynamic and conventional vines. *Scientific Reports*, *8*, 16857.

Tenenhaus, M., Esposito Vinzi, V., Chatelinc, Y. & Lauro, C. (2005) PLS path modeling. *Computational Statistics & Data Analysis*, *48*, 159–205.

Welter, L.J., Göktürk-Baydar, N., Akkurt, M., Maul, E., Eibach, R., Töpfer, R. et al (2007) Genetic mapping and localization of quantitative trait loci affecting fungal disease resistance and leaf morphology in grapevine (*Vitis vinifera* L). *Molecular Breeding*, *20*, 359–374.

Welter, L.J., Tisch, C., Kortekamp, A., Töpfer, R. & Zyprian, E. (2017) Powdery mildew responsive genes of resistant grapevine cultivar 'Regent'. *Vitis*, *56*, 181–188.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Calonnec A, Jolivet J, Ramaroson M-L, Dufour M-C, Corio-Costet M-F. Defence responses of grapevine cultivars to powdery mildew: Ontogenic resistance versus genetic resistance. *Plant Pathol*. 2021;00:1–18. https://doi.org/10.1111/ppa.13404