RESEARCH PAPER

Transcriptome and metabolome reprogramming in *Vitis vinifera* cv. Trincadeira berries upon infection with *Botrytis cinerea*

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Abstract

Vitis vinifera berries are sensitive towards infection by the necrotrophic pathogen *Botrytis cinerea*, leading to important economic losses worldwide. The combined analysis of the transcriptome and metabolome associated with fungal infection has not been performed previously in grapes or in another fleshy fruit. In an attempt to identify the molecular and metabolic mechanisms associated with the infection, peppercorn-sized fruits were infected in the field. Green and veraison berries were collected following infection for microarray analysis complemented with metabolic profiling of primary and other soluble metabolites and of volatile emissions. The results provided evidence of a reprogramming of carbohydrate and lipid metabolisms towards increased synthesis of secondary metabolites involved in plant defence, such as *trans*-resveratrol and gallic acid. This response was already activated in infected green berries with the putative involvement of jasmonic acid, ethylene, polyamines, and auxins, whereas salicylic acid did not seem to be involved. Genes encoding WRKY transcription factors, pathogenesis-related proteins, glutathione S-transferase, stilbene synthase, and phenylalanine ammonia-lyase were upregulated in infected berries. However, salicylic acid signalling was activated in healthy ripening berries along with the expression of proteins of the NBS-LRR superfamily and protein kinases, suggesting that the pathogen is able to shut down defences existing in healthy ripening berries. Furthermore, this study provided metabolic biomarkers of infection such as azelaic acid, a substance known to prime plant defence responses, arabitol, ribitol, 4-amino butanoic acid, 1-O-methyl-glucopyranoside, and several fatty acids that alone or in combination can be used to monitor *Botrytis* infection early in the vineyard.

Key words: *Botrytis cinerea*, grapevine, grape ripening, metabolome, plant defence, transcriptome.

Introduction

Grapes (*Vitis* spp.) are economically the most important fruit crop worldwide with a global production of around 67 million t in 2012 (Food and Agriculture Organization Corporate Statistical Database (FAOSTAT, 2014, <http://faostat.fao.org/>

Abbreviations: EI, electron impact ionization; FDR, false discovery rate; GC, gas chromatography; LRR, leucine-rich repeat; MS, mass spectrometry; NBS, nucleotide-binding site; PCA, principal component analysis; R, resistance; SE, standard error; SPME, solid-phase microextraction; TOF, time-of-flight.

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site/567/default.aspx#anchor). Moreover, the consumption of table grapes and wine has numerous nutritional and health benefits for humans due to antioxidant polyphenols such as resveratrol (Yadav *et al.*, 2009).

Grapevines are prone to several diseases, with fungi being the major cause of damage and losses in grape quality and yields, consequently affecting wine production worldwide. Despite research efforts in understanding pathogen susceptibility/resistance in grapevine, the mechanisms that regulate these processes remain unclear although a number of insights have been published (Verhagen *et al.*, 2010; Feechan *et al.*, 2013; Nanni *et al.*, 2013). The first resistance genes (TIR-NB-LRR genes), which confer resistance to powdery and downy mildews in the grapevine species (*Muscadinia rotundifolia*), have recently been cloned (Feechan *et al.*, 2013). Moreover, VvAMP2, a grapevine defensin expressed in flowers, was shown to be active against the fungal pathogen *Botrytis cinerea* (Nanni *et al.*, 2013).

Trincadeira is a very important Portuguese grapevine cultivar, giving rise to unique and excellent wines in certain years, but is extremely susceptible to fungal pathogens such as grey mould caused by *B. cinerea* Pers.:Fr, which is one of the most dramatic grape diseases. The presence of this necrotrophic fungus in the grapevines causes severe reductions in both quality and quantity of grapes and wine as a consequence of modifications in the chemical composition of the grape berry itself (Bocquet and Valade, 1995). Recently, significant changes in the metabolome of fully mature berries infected with *B. cinerea* were reported using an ¹H-nuclear magnetic resonance -based platform (Hong *et al.*, 2012), although only 23 metabolites were identified in this study. Modifications of the global metabolite profiles were reported in champagne-base wines when vinified with *B. cinerea*-infected grapes, thus revealing an inhibition of the wine fermentation and a subsequent quality loss (Hong *et al.*, 2011). *Botrytis* complicates winemaking by producing compounds that kill yeast and often results in fermentation stopping before the wine has accumulated sufficient levels of alcohol.

Necrotrophs kill their host cells by secreting toxic compounds or lytic enzymes and produce an array of pathogenicity factors that can subdue host defences (van Kan, 2006). Grape clusters can be infected with *B. cinerea* at bloom and after veraison, depending on climatic conditions and the sensitivity of the grape variety. Between bloom and veraison, grape berries were reported to be resistant against infections occurring in nature, partly due to a high concentration of specific phenolic compounds (Goetz *et al.*, 1999), although they can harbour the pathogen as endophytic without any visible signs of disease development. Despite elaborate research studies, the biochemical and genetic basis of resistance to *Botrytis* is still not fully understood. Constitutive and inducible plant secondary metabolites and a complex interplay between different plant hormones regulate defence gene expression and disease resistance (Van Baarlen *et al.*, 2004; Glazebrook, 2005; Blanco-Ulate *et al.*, 2013). During *Arabidopsis* infection, subsets of the *Botrytis*-induced genes are regulated by functional jasmonate and ethylene signalling pathways and three *Botrytis*-induced transcription factors are

sensitive to abscisic acid (AbuQamar *et al.*, 2006). Moreover, an abscisic acid-deficient tomato mutant was found to present resistance to *Botrytis* and to accumulate hydrogen peroxide earlier and more strongly than the susceptible wild type (Asselbergh *et al.*, 2007).

Understanding the complexity of the mechanisms responsible for fruit susceptibility to infection and how this affects wine characteristics is critically important to improve grape-growing practices and wine quality in a global and competitive market with limited use of fungicides. Transcriptomic and metabolomic studies have unravelled some mechanisms underlying the ripening of grapes (Deluc *et al.*, 2007; Grimplet *et al.*, 2007; Pilati *et al.*, 2007; Fortes *et al.*, 2011; Agudelo-Romero *et al.*, 2013b) and grapevine responses of susceptible and resistant varieties towards fungal infection (Figueiredo *et al.*, 2008; Fung *et al.*, 2008; Polesani *et al.*, 2010; Hong *et al.*, 2012). However, no previous work has addressed the parallel analysis of the transcriptome and metabolome of infected fruits. This is also the first transcriptomic study of the response to *B. cinerea* in grapevine berries supported by a significant coverage of the metabolites associated with the infection at precocious stages. Thus, our study provides novel information regarding biotic stress responses in grapes as well as new insights into how fungal diseases, in particular *B. cinerea*, may affect host metabolism and the regulation of berry ripening. Furthermore, the initiation of defence mechanisms against necrotrophic, biotrophic and hemi-biotrophic pathogens has been documented mostly for vegetative tissues (Blanco-Ulate *et al.*, 2013). With this work, we bring novel insights into the responses of fruits during a pathogen–host interaction by a systems analysis of the transcriptome and metabolome.

Materials and methods

Fungal infection and sample collection

Field experiments were conducted in an experimental vineyard with 15-year-old grapevines (*Vitis vinifera* L. cv. Trincadeira) at the Instituto Superior de Agronomia, University of Lisbon, Portugal. Vines were cane pruned to two buds and trained to the Lys training system (3.0×1.0). The *B. cinerea* isolate used was obtained from diseased grapevine plants and maintained in potato dextrose agar (Difco, Detroit, MI, USA), at 5 °C. Conidia production was achieved by exposing inoculated Petri dishes with potato dextrose agar to continuous fluorescent light, at 24 °C. Conidia were harvested from 14- to 20-day-old cultures and collected by rubbing with phosphate buffer (0.03 M KH₂PO₄), filtered through cheesecloth to remove mycelia, and the concentration adjusted to 10⁵ conidia ml⁻¹.

Infections were made on berries by spraying the conidial suspension at the developmental stage of peppercorn size (EL29). Control clusters were sprayed with phosphate buffer. Following inoculation, the clusters were incubated by enclosing them in individual plastic bags for a period of 1 week. Collection of samples was performed at two stages of development at pre-veraison (EL33) and veraison (EL35) (Coombe, 1995). For each time point and each treatment (infected and mock), four biological replicates were collected on 30 June and 12 July 2011. All the berries from infected and healthy (control) clusters were harvested around 10 a.m., immediately frozen in liquid nitrogen, and transported to the laboratory in dry ice and kept at –80 °C. Prior to extraction for transcriptomic and metabolomic analyses, the seeds were removed. Three biological replicates

were used for microarray experiments and four biological replicates were used for metabolomics.

RNA extraction

RNA extraction was carried out according to Fortes *et al.* (2011). A DNase treatment was performed according to the supplier's instructions (Invitrogen, San Diego, CA, USA). RNA was further purified using an RNeasy Plant Mini kit (Qiagen, Valencia, CA, USA).

Microarray hybridization and data extraction

RNA quantity and quality were determined using a Nanodrop 2000 instrument (Thermo Scientific, Wilmington, DE, USA) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). The cDNA synthesis, labelling, hybridization, and washing reactions were performed according to the NimbleGen Arrays User's Guide (v.3.2). cDNA was synthesized from 10 µg of total RNA using a cDNA Synthesis System kit (Roche NimbleGen). The cDNA preparation (1 µg) was amplified and labelled with Cy3-labelled random nonamers using a One Colour Labelling kit (Roche NimbleGen). If the quality control was correct, then 4 µg of labelled cDNA was hybridized on a NimbleGen microarray 090818 Vitis exp HX12 (Roche NimbleGen), representing 29 549 predicted genes on the basis of the grapevine 12X V1 gene prediction version [platform accession number in Gene Expression Omnibus (GEO) database: GPL17894]. Hybridization solution (NimbleGen Hybridization kit) was added to each labelled cDNA and hybridization was performed for 16 h at 42 °C in a HS 4 Hybridization station (Roche NimbleGen). Hybridized microarrays were washed with a Wash Buffer kit (Roche NimbleGen) and scanned at 532 nm and 2 µm resolution in a DNA Microarray Scanner with SureScan High-Resolution Technology (Agilent Technologies).

After evaluation of hybridization quality by an experimental metrics report implemented in NimbleScan Software v.2.6 (Roche NimbleGen), probeset signal values from all the microarray hybridizations were corrected for background and normalized using Robust Microarray Average (RMA) (Irizarry *et al.*, 2003) in NimbleScan Software, which produces a call file for each sample with normalized expression data for each gene.

The robustness of datasets was evaluated using Pearson correlation (which ranged between 0.956 and 0.945%). A Bayes *t*-statistics from the linear models for microarray data (limma) (Smyth, 2004) was run in FIESTA (Oliveros, 2007) for differential expression analysis, and *P* values were corrected for multiple testing using the Benjamini and Hochberg (1995) method. Differentially expressed genes were identified considering a false discovery rate (FDR) of ≤5% and fold change of ≥2.0 or ≤−2.0. In certain cases, where integration with metabolomics data was presented, a fold change of ≥1.5 or ≤−1.5 was also considered. The data presented in this study have been deposited in the NCBI Gene Expression Omnibus and are accessible through GEO Series accession number GSE52586. Principal component analysis (PCA) was performed with the full RMA dataset using Acuity 4.0 (Axon Molecular Devices, <http://www.moleculardevices.com>).

Functional enrichment analysis

Gene lists were analysed further with FatiGO (Al-Shahrour *et al.*, 2004) to identify significant functional enrichment following a grapevine-specific functional classification of 12X V1 predicted transcripts (Grimple *et al.*, 2012). Fisher's exact test was carried out in FatiGO to compare each study list with the list of total non-redundant transcripts housed in the grapevine 12X V1 gene predictions (Grimple *et al.*, 2012). Significant enrichment was considered for *P* < 0.05 after Benjamini and Hochberg correction for multiple testing.

Real-time PCR

First-strand cDNA was synthesized from 2 µg of total RNA as described previously (Fortes *et al.*, 2011). Real-time PCRs were

performed using the StepOne™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Cycling conditions were 95 °C for 10 min, followed by 42 cycles of 95 °C for 30 s and 60 °C for 45 s. Relative expression data were derived from three biological replicates and duplicate technical replicates. The standard curve was built using a serial dilution of mixtures of all cDNAs analysed. Data were normalized using the expression curves of the actin gene (VIT_04s0044g00580) and elongation factor 1α gene (VIT_06s0004g03220). These genes are the most stable according to NormFinder software (data not shown). All primers used are shown in Supplementary Table S1 at JXB online.

Soluble metabolites

The profiling of soluble metabolites was performed as detailed previously (Wagner *et al.*, 2003; Erban *et al.*, 2007) by gas chromatography coupled to electron impact ionization/time-of-flight mass spectrometry (GC-EI/TOF-MS) using an Agilent 6890N24 gas chromatograph (Agilent Technologies).

Soluble metabolites were extracted from 300 mg (fresh weight ± 10 % tolerance) deep-frozen powder as described previously (Agudelo-Romero *et al.*, 2013b).

GC-EI/TOF-MS chromatograms were acquired, visually controlled, baseline corrected, and exported in NetCDF file format using ChromaTOF software (v.4.22; LECO, St Joseph, USA). GC-MS data processing into a standardized numerical data matrix and compound identification were performed using the TagFinder software (Luedemann *et al.*, 2008; Allwood *et al.*, 2009). Compounds were identified by mass spectral and retention time index matching to the reference collection of the Golm metabolome database (GMD, <http://gmd.mpimp-golm.mpg.de/>) (Kopka *et al.*, 2005; Hummel *et al.*, 2010). Guidelines for manually supervised metabolite identification were the presence of at least three specific mass fragments per compound and a retention index deviation of <1.0% (Strehmel *et al.*, 2008). The measured intensities of all mass features of an experiment were divided by the measured intensity of the mass features representing the internal standard (*C*₂₂) to account for volume errors during GC analysis and in addition divided by sample fresh weight (g) to account for variations in the sampled masses in order to obtain normalized responses.

Volatile metabolites

The profiling of volatile metabolites was evaluated using 1 g (fresh weight ± 10 % tolerance) as described previously (Agudelo-Romero *et al.*, 2013b). Analyses were performed by solid-phase microextraction (SPME) and GC coupled to EI/quadrupole MS (GC-EI/QUAD-MS) using an Agilent 6890N24 gas chromatograph (Agilent Technologies) and a StableFlex™ SPME-fiber with 65 µm polydimethylsiloxane/divinylbenzene coating (Supelco, Bellefonte, USA). GC-MS data processing into a standardized numerical data matrix and compound identification were performed as described above using the TagFinder software (Luedemann *et al.*, 2008; Allwood *et al.*, 2009). Compounds were identified by mass spectral and retention time matching to the reference collection of the Golm Library for volatile compounds. Guidelines for manually supervised metabolite identification were the presence of at least three specific mass fragments per compound and a retention time deviation of <3.0%.

Relative quantification of metabolite concentrations

For relative quantification purposes, all mass features were evaluated for best specific, selective, and quantitative representation of observed analytes. Laboratory and reagent contaminations were evaluated by non-sample control experiments. Metabolites were routinely assessed by relative changes expressed as response ratios, i.e. *x*-fold factors of normalized responses in comparison to a control condition (Supplementary Table S3 at JXB online).

Statistical analyses and data visualization

Statistical testing was performed using $\log_{10}$ -transformed response ratios. Statistical assessments and heat mapping were performed with the multi-experiment viewer software MeV (v.4.7.4; <http://www.tm4.org/>; Saeed *et al.*, 2003) or the Microsoft Excel 2010 program. Independent component analysis of the first five principal components was performed using $\log_{10}$ -transformed response ratios via the MetaGeneAlyse web application (v.1.7.1; <http://metagene-alyse.mpimp-golm.mpg.de>). Excel files containing ion peak responses were analysed by Student's *t*-test and one-way and two-way analysis of variance, as well as by the Kruskal–Wallis and Wilcoxon rank sum tests (Supplementary Table S3). Metabolic markers were considered the compounds present in infected samples but absent in control samples as well as those metabolites that presented a very significant increase in infected samples.

Anthocyanin quantification

Anthocyanin concentration was measured as described previously (Waters *et al.*, 2005) with modifications. Lyophilized material (20–60 mg) was extracted in 1 ml of methanol for 1 h at 4 °C. The mixture was then centrifuged for 30 min at 13 000 rpm at 4 °C. Afterwards, 100 μ l of this sample was diluted into 1 ml of methanol. Total relative anthocyanin concentration was expressed as the absorbance value at 520 nm g^{-1} of freeze-dried weight.

Results and discussion

Phenotypic and metabolic characterization of infected and mock-treated grape berries

Berries were inoculated with *B. cinerea* conidial suspension at the peppercorn-sized fruits stage corresponding to developmental stage EL29 according to the Coombe (1995) classification. The first symptoms of infection were observed at the precocious stage of EL31 (Supplementary Fig. S1 at JXB online). The developmental stages investigated in this study were identified as EL33 characterized by hard green berries accumulating organic acids and EL35 corresponding to

veraison when anthocyanin accumulation initiates. Figure 1 shows an infected cluster corresponding to EL33 in which high contamination with *B. cinerea* was observed. Evaluation of sample infection was performed by visual inspection (Supplementary Table S2 at JXB online) and additionally by PCR using primers specific to the fungal genomic DNA (Supplementary Fig. S2 at JXB online). The assessment took into account the berry weight and the content of the main organic acids, sugars, and anthocyanins present in healthy berries (Fig. 2). The effects of *B. cinerea* infection were a significant decrease in berry weight and organic acids in infected compared with non-infected samples and an increase in anthocyanin, fructose, and glucose contents, indicating that infected berries were riper than non-infected berries, in particular at the EL35 stage.

Metabolic profiling of grapes was carried out using a GC-EI-TOF/MS platform that enabled the relative quantification of several classes of compounds such as fatty acids, phenylpropanoids, and sugars among the polar compounds (Supplementary Table S3). Additional metabolic profiling was achieved using a headspace GC-EI-MS platform for the relative quantification of volatile emissions.

The normalized responses were used for multivariate data analysis using the unsupervised method of independent component (IC) analysis. A good discrimination was obtained between control and infected samples with five principal components, which accounted for 80% of variance (Fig. 3C). The resulting IC1 discriminated infected from mock-treated samples, whereas IC3 differentiated developmental stages (EL33 and EL35).

To confirm whether metabolites were present in significantly different amounts during ripening of both infected and control samples, several statistical tests were performed also using normalized responses (Supplementary Table S3). These tests showed that the majority of metabolites (67.5%)

Fig. 1. Cluster of Trincadeira grapes at EL33 infected with *B. cinerea*. (A) Sporulation of the fungus was already observed by this stage in some berries of the cluster. (B) Magnification of (A). (This figure is available in colour at JXB online.)

Fig. 2. Phenotypic and metabolic characterization of infected and mock-treated grape berries. (A, B) Fresh berry weight (A; g) and total anthocyanin content (B; absorbance at 520 nm g^{-1} of freeze-dried material) of infected and control berries at developmental stages of EL33 and EL35. Bars represent the standard error (SE). Statistically significant results are indicated: *, $P < 0.05$; +, $P < 0.1$. (C) Relative quantification of malic acid, tartaric acid, fructose, and glucose based on normalized responses. Specific chromatogram ion peaks were selected for relative quantification (based on peak height). Malate and tartrate content decreased in infected berries, whereas the content of fructose and glucose increased in these samples. Bars represent SE.

exhibited a different content comparing either between developmental stages or between control and infected samples. Among these, 24.2% were positive markers of infection (Figs 3B and 4).

The differential accumulation of metabolites was represented by a heat map (Fig. 3A and Supplementary Fig. S3 at JXB online). Fatty acids accumulated in infected berries at EL33 and EL35, whereas sugars (glucose and fructose) increased in

infected berries only at EL35. Some of these fatty acids and sugars were also veraison responsive (Fig. 3A and Supplementary Fig. S4 at JXB online). Certain organic acids such as malic, glyceric, threonic, and lactic acid decrease in infected berries together with the aromatic benzoic acid (Supplementary Fig. S5 at JXB online). Shikimic acid, a precursor of phenylpropanoids, and β -sitosterol increased at EL35 in infected berries. This steroid was also a veraison-responsive metabolite and has

Fig. 3. Analysis of infection- and veraison-responsive metabolites from developing berries of *V. vinifera* cv. Trincadeira. Soluble and volatile metabolites were profiled at developmental stages EL33 and EL35, i.e. veraison, after *B. cinerea* infection and were compared with non-infected berries. (A) Metabolites that were significantly increased or decreased in at least one of the comparisons ($P < 0.05$, Wilcoxon test; *, significant). Response ratios were log₂ transformed and hierarchically clustered using Euclidian distance and complete linkage. Five clusters (CL_I–CL_V) represent the main response patterns of the monitored metabolites (Supplementary Table S3). (B) Venn diagram of infection- and veraison-responsive metabolites. (C) Independent component (IC) analysis of metabolite profiles at stages EL33 and EL35 with and without *Botrytis* infection. Five principal components representing 80% of the total variance were used to calculate independent components. The plot shows the sample scores of IC1 and IC3. (This figure is available in colour at JXB online.)

been indicated to be involved in plant–pathogen interactions (Griebel and Zeier, 2010). Gallic acid and 3,4-dihydroxybenzoic acid increased tremendously at EL33 and EL35 in infected berries together with the phenylpropanoid *trans*-resveratrol (Figs 3A and 4A). Another phenylpropanoid showing differential content was *trans*-4-hydroxy-cinnamic acid, which increased at EL33 in infected berries. The triterpenoid oleanolic acid decreased in infected berries at EL33 and EL35, although it increased at veraison during regular ripening. These secondary metabolites are likely to play a role in grape defence responses, because their biosynthesis is either activated or inhibited in

infected berries compared with healthy berries. Regarding volatiles, few metabolites presented a differential content among the samples except for 1,4-dichlorobenzene and acetone, which increased in infected berries at EL33 and EL35, respectively. In addition, acetone, (Z)-3-hexen-1-ol and phenylacetaldehyde were veraison-responsive metabolites.

Concerning infection-related metabolism, 28 metabolites were identified as markers of infection (Fig. 4) covering acids such as gallic and azelaic acid, amino acids (4-amino-butanoic acid), several fatty acids, lipids (α -tocopherol), phenylpropanoids (*trans*-resveratrol), polyols such as arabinol,

Fig. 4. Analysis of potential positive metabolic markers of *B. cinerea* infection at stages EL33 and EL35 (veraison) of developing berries of *V. vinifera* cv. Trincadeira. (A) Metabolites that were significantly increased in both comparisons ($P < 0.05$, Wilcoxon test) and metabolites that became detectable after infection were selected. Grey boxes indicate that the respective metabolites were not detectable. (B) Normalized response plot of selected metabolites that accumulated after infection. (This figure is available in colour at JXB online.)

ribitol, and *myo*-inositol, and sugars such as 1-*O*-methylglucopyranoside, revealing the existence of a substantial metabolic reprogramming upon infection.

Transcriptional and functional enrichment analyses of infected and mock-treated grape berry samples

The mRNA expression profiles of infected and healthy berries at two developmental stages (EL33 and EL35) were compared using the NimbleGen microarray 090818 Vitis exp

HX12 chip. As a first approach to analyse the complexity of the gene expression dataset, a PCA was performed. The first and second principal components (PC1 and PC2) explained together 64.3% of the variability in gene expression (44.16 and 20.14%, respectively) (Supplementary Fig. S6 at JXB online). The total number of unique IDs that were differentially expressed either due to the infection and/or due to ripening was 9169 (Fig. 5A and Supplementary Table S4 at JXB online). From these, 4437 were differentially expressed upon infection.

Fig. 5. Venn diagrams and functional analysis of differentially expressed genes. (A) Venn diagrams of significant transcripts (5% FDR in limma and ≥ 2 -fold change; [Supplementary Table S4](#)) comparing infection-responsive transcripts between EL33 and EL35 berry developmental stages (left diagram) as well as veraison-responsive transcripts between infected and control berries (right diagram). (B) Summary of functional categories ([Grimplet et al., 2012](#)) significantly enriched (5% FDR in a Fisher's exact test) within infection up- and downregulated transcripts in each developmental stage. Categories appear in order of significance. The whole list of significant categories can be found in [Supplementary Table S5](#) at JXB online. (This figure is available in colour at JXB online.)

Functional enrichment analysis of up- and downregulated transcripts on each comparison was carried out to assess their underlying biological significance ([Fig. 5](#) and [Supplementary Table S5](#)).

We observed among genes upregulated in infected berries at EL33 an over-representation of genes involved in secondary metabolism (stilbenoid, anthocyanin, phenylalanine, and phenylpropanoid biosynthesis) and primary metabolism (α -linolenic acid, tyrosine, and amino sugar metabolisms in infected EL33 berries as well as an enrichment in galactose and trehalose metabolisms in infected EL35 berries), suggesting that infection leads to a reprogramming of primary and secondary metabolism putatively related to defence activation. In addition, we observed activation of stress responses involved in infection and defence processes such as the oxidative stress response (EL33), desiccation stress response (EL33 and EL35) and heat-shock proteins (EL35), most likely related to the dehydration effects caused by *B. cinerea* infection ([Vannini and Chilosi, 2013](#)). These responses could be

regulated by auxin-, ethylene-, and jasmonic acid-mediated signalling pathways together with the WRKY, AP2, and MYB families of transcription factors ([Fig. 5](#)). At EL35 in infected berries, we also observed enrichment in xyloglucan modification, suggesting that cell-wall remodelling occurs as part of the response to pathogen attack. Altogether, these results indicated that infected berries initiate a defence response.

On the other hand, among genes downregulated in infected berries at EL33 and/or EL35, there was over-representation of classes involved in the biotic stress response including R proteins from plant-pathogen interactions, the NBS-LRR superfamily, sphingolipid metabolism and glycerolipid catabolism, cytoskeleton organization, and biogenesis, among others, suggesting the possible effect of *B. cinerea* in shutting down specific pathogen responses and the reduction of specific lipid metabolism. Later in infection, energy metabolism (photosystem I supercomplex) and secondary metabolism (phenylpropanoid and stilbenoid biosynthesis) also seemed to be downregulated. In addition, downregulated genes at

EL35 in infected berries were also enriched in the classes of cytochrome P450, ERF subfamily of transcription factors, protein kinases, jasmonic acid- and brassinosteroid-mediated signalling pathways, and subtilase-mediated proteolysis, indicating that specific defence responses were inhibited due to ripening. Moreover, flavonoid, isoflavonoid, and sesquiterpenoid biosynthesis was enriched at EL35. On the other hand, salicylic acid signalling, the NBS-LRR superfamily, and protein kinase were enriched at EL35 in healthy berries (Supplementary Table S5). Altogether, the results suggested that *B. cinerea* induces a suppression of certain developmentally activated defences with alterations in the normal ripening programme and fruit composition.

To validate the microarray data, real-time quantitative (q)PCR analysis was performed for six selected genes (Supplementary Fig. S7 at JXB online), revealing an agreement between both data sets (Table 1 and Supplementary Table S4).

Combined analysis of gene expression and metabolite data

Biotic stress responses differentiate infected and healthy berries and involve selective activation of secondary metabolism

One of the most remarkable responses observed at the transcriptional level was the alteration of defence-related responses. One of the functional categories enriched in down-regulated infection-responsive transcripts was the NBS-LRR superfamily (VIT_04s0008g00320 and VIT_18s0041g01700; Fig. 5, Table 1, and Supplementary Table S4). The genes encoding the specificity determinants of effector-triggered immunity are known as resistance (R) genes. Most R genes encode proteins that contain a nucleotide-binding site (NBS) and leucine-rich repeats (LRRs). NBS-LRR proteins play a role in pathogen sensing and host defence (DeYoung and Innes, 2006). At EL33 in infected berries, there was also down-regulation of multiple genes encoding R proteins from plant–pathogen interactions (VIT_00s0335g00090 and VIT_18s0041g01620). R-gene-mediated specificity of plant–pathogen recognition is not documented for necrotrophic pathogens (Bent and Mackey, 2007), so it seems that defence responses that were not specific against *Botrytis* were down-regulated. In fact, the primary genetic determinants of effector-triggered immunity and the related immune responses are not effective regulators of resistance to necrotrophs (Mengiste *et al.*, 2012).

Some of these genes were induced at veraison in healthy berries during regular ripening (Supplementary Tables S4 and S5) along with other genes involved in plant–pathogen interactions, the biotic stress response, and protein kinases (VIT_05s0077g01600, VIT_06s0004g01000, and VIT_09s0002g03020). Plant receptor kinases are involved in plant–microbe interactions (Haffani *et al.*, 2004). Several genes encoding Clavata1 receptor kinase (CLV1) were down-regulated with the infection but up-regulated at veraison (VIT_04s0008g00310; Supplementary Table S4), as confirmed by qPCR (Supplementary Fig. S7). Interestingly,

the *XA-21* gene from rice, which provides resistance to *Xanthomonas oryzae* pv. *oryzae* race 6, encodes a protein identical in overall structure to CLV1 (Song *et al.*, 1995). The same was verified for genes encoding Wall-associated kinase 1 (WAK1) (VIT_03s0132g00390; Table 1). Overexpressing WAK1 enhanced resistance to *B. cinerea* in *Arabidopsis* (Brutus *et al.*, 2010). This indicates that many defences that are naturally developed by healthy ripe berries are not present in infected berries, suggesting that the pathogen is able to shut down part of the transcriptional ripening programme.

In addition, some genes involved in phenylpropanoid and isoflavonoid biosynthesis that may play a role in biotic stress response were expressed at veraison in healthy grapes but were absent in infected grapes (VIT_12s0028g02810 and VIT_09s0096g00520). The same was verified for terpenoids, in particular for triterpenoid and sesquiterpenoid biosynthesis (VIT_09s0054g01520 and VIT_18s0001g04490). Interestingly, oleanolic acid or oleanic acid, a triterpenoid, was decreased in infected grapes at EL33 and EL35 although it is a veraison-responsive metabolite (Fig. 3 and Supplementary Fig. S4). Unconjugated triterpenoids, such as oleanolic acid, are often found in the epicuticular waxes of plants, serving as a first-defence barrier against pathogens (Heinzen *et al.*, 1996).

However, some defences were mounted in infected berries that were also expressed at veraison in healthy berries (Fig. 5 and Supplementary Table S5), such as increased stilbenoid and phenylpropanoid biosynthesis and the biotic stress response (VIT_16s0100g00770, VIT_16s0100g01070, VIT_06s0004g01030, and VIT_04s0008g02070). In grapevine, stilbenes such as resveratrol are phytoalexins active against downy and powdery mildew and grey mould (Chang *et al.*, 2011; Malacarne *et al.*, 2011). At EL33, *trans*-resveratrol was only detected in infected grapes (Fig. 4). The resistance of *Vitis* spp. to *B. cinerea* infection was indeed shown to correlate with *trans*-resveratrol content (Gabler *et al.*, 2003).

Stilbenoid biosynthesis, including several stilbene synthases, was also enriched at EL33 in infected berries (Fig. 5) in good agreement with increased *trans*-resveratrol levels, suggesting that at EL33 a more intense activation of defences occurs (Fig. 6 and Supplementary Fig. S8 at JXB online). We observed at EL33 in infected berries enrichment in phenylalanine and phenylpropanoid biosynthesis with several genes encoding phenylalanine ammonia-lyase being up-regulated (VIT_00s2508g00010 and VIT_16s0039g01130; Supplementary Fig. S8). Modification of the plant cell wall by the incorporation of phenolic compounds is an important defence mechanism against *B. cinerea* (Curvers *et al.*, 2010; Bassolino *et al.*, 2013). Therefore, fruits react towards the synthesis of secondary metabolites. However, phenylpropanoid compounds that are accumulated as a response to fungal infection (Lima *et al.*, 2010) were completely degraded in botrytized berries at the harvest stage (Hong *et al.*, 2012). Our assessment of phenylpropanoids was limited to resveratrol, catechin, and *trans*-4-hydroxycinnamic acid. This latter compound increased in infected berries at EL33 (Fig. 3). It may be that at earlier stages of grape development the above-mentioned degradation was not yet pronounced.

Table 1. Selection of genes differentially expressed in infected and control grapes at EL33 and EL35 (considering an FDR of ≤5% and fold change of ≥2 or ≤−2)

The comparisons of EL33 infected versus EL33 mock, EL35 infected versus EL35 mock, EL35 infected versus EL33 infected, and EL35 mock versus EL33 mock are provided.

Unique ID	Fold change				Functional annotation
	EL33Inf/ EL33Mock	EL35Inf/ EL35Mock	EL35Inf/ EL33Inf	EL35Mock/ EL33Mock	
Biotic stress response and secondary metabolism					
VIT_00s0335g00090	−2.35		2.52		R protein MLA10
VIT_00s1455g00010	3.00	2.86			Expansin β1 precursor
VIT_00s2508g00010	7.40		−2.22	6.02	Phenylalanine ammonia-lyase
VIT_02s0012g01570	2.49	2.79	2.42	2.17	Cinnamoyl-CoA reductase
VIT_02s0025g00260	2.90		−2.31		Polygalacturonase GH28
VIT_03s0132g00390		−3.50		3.06	Wall-associated kinase 1 (WAK1)
VIT_04s0008g00310		−9.87	−2.31	3.47	Clavata1 receptor kinase (CLV1)
VIT_04s0008g00320		−9.69		4.46	Leucine-rich repeat family protein
VIT_04s0008g02070	2.40	−2.77	−2.79	2.38	Avr9/Cf-9 induced kinase 1
VIT_05s0077g01600		−3.78		3.47	Pathogenesis protein 10 (<i>Vitis vinifera</i>)
VIT_05s0094g00360	4.36			3.02	Chitinase class IV
VIT_06s0004g01000		−2.66		2.44	Dirigent protein
VIT_06s0004g01030	7.76			11.44	Dirigent protein
VIT_06s0004g07040	2.04		−2.55		Glutaredoxin
VIT_09s0002g03020		−3.94		3.25	FRK1 (FLG22-induced receptor-like kinase 1)
VIT_09s0054g01520		−2.58		2.11	β-Amyrin synthase
VIT_09s0070g00240	2.09	−3.77	−4.16		Cinnamoyl-CoA reductase
VIT_09s0096g00520		−3.47		2.19	Coniferyl alcohol acyltransferase
VIT_12s0028g02810		−8.79		6.21	Isoflavone methyltransferase/orcinol O-methyltransferase 1 oomt1
VIT_16s0039g01130	9.35		−2.31	6.64	Phenylalanin ammonia-lyase (<i>Vitis vinifera</i>)
VIT_16s0100g00770	9.07	−2.28	−2.53	8.17	Stilbene synthase
VIT_16s0100g01070	10.24	−2.33	−2.34	10.20	Resveratrol (<i>Vitis vinifera</i>)
VIT_18s0001g04490		−15.89		19.23	Germacrene-D synthase
VIT_18s0041g01620	−2.47				R protein L6
VIT_18s0041g01700	−2.36		3.80		TIR-NBS-LRR-TIR disease resistance protein
VIT_19s0093g00390	2.55		−3.41		Glutathione S-transferase 25 GSTU25
Hormone metabolism					
VIT_03s0063g01820	15.41	17.89	2.10		AOS (allene oxide synthase)
VIT_04s0008g03400	2.32				DREB sub A-4 of ERF/AP2 transcription factor
VIT_05s0020g03200		11.69	7.65	−2.17	Spermine synthase
VIT_06s0004g07500	2.18				WRKY DNA-binding protein 33
VIT_12s0059g01380	2.72			2.42	1-aminocyclopropane-1-carboxylate oxidase 1
VIT_13s0064g00960	2.04				Myb domain protein 46
VIT_15s0046g02220	4.41		−2.74		ACC synthase
VIT_16s0013g01110	2.13				Ethylene-responsive transcription factor 5
VIT_17s0000g07370		−4.10		3.35	EDS1 (Enhanced disease susceptibility 1)
VIT_17s0000g07420		−4.39		3.62	EDS1 (Enhanced disease susceptibility 1)
VIT_18s0001g00740	3.03		−2.20		Ornithine decarboxylase
VIT_18s0001g10500	−2.54		−2.78	−5.34	ABA 8'-hydroxylase CYP707A1
VIT_18s0001g11630	2.03				Allene oxide synthase
VIT_18s0041g02070	2.15		−4.20		12-Oxophytodienoate reductase 1
Carbohydrate metabolism					
VIT_00s0181g00180		−2.08	−4.98	−3.15	LHCB3 (light-harvesting chlorophyll binding protein 3)
VIT_00s0270g00120	45.38	4.58	−9.30		α-Amylase/subtilisin inhibitor
VIT_00s0270g00120	45.38	4.58	−9.30		α-Amylase/subtilisin inhibitor
VIT_01s0010g02460	2.02			2.15	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic 3
VIT_01s0127g00260		2.97	3.91		ATP-citrate synthase
VIT_02s0154g00110	2.33	3.68	2.19		Trehalose-6-phosphate phosphatase (AtTPPA)
VIT_03s0038g00670		−2.09	−3.16		Fructose-bisphosphate aldolase, chloroplast precursor
VIT_04s0044g01410		−2.95	−5.70	−2.13	Photosystem I reaction centre subunit N (PSAN)
VIT_05s0029g01140	−4.50		4.72		Sucrose-phosphate synthase

(continued)

Table 1. Continued

Unique ID	Fold change				Functional annotation
	EL33Inf/ EL33Mock	EL35Inf/ EL35Mock	EL35Inf/ EL33Inf	EL35Mock/ EL33Mock	
VIT_05s0077g00840	5.21	5.51			Galactinol-raffinose galactosyltransferase
VIT_07s0005g01680	2.42	3.77			Stachyose synthase
VIT_12s0059g02350	4.20		−3.00		Isocitrate lyase (isocitrase) (isocitratase) (ICL)
VIT_14s0060g00790	8.31	5.03			Galactinol synthase
VIT_14s0066g00810	3.88	4.83			Raffinose synthase
VIT_17s0000g01820	3.03	8.40	3.21		Malate synthase, glyoxysomal
VIT_17s0000g08010	2.15			3.67	Trehalose 6-phosphate synthase
VIT_17s0119g00280	5.10		−4.17		α-Amylase/subtilisin inhibitor
<i>Lipid metabolism</i>					
VIT_01s0011g04380		2.18			Acyl-(acyl carrier protein) thioesterase
VIT_03s0063g01880	−3.34	2.52	6.43		Acyl-CoA synthetase long-chain member 2
VIT_04s0008g02250		2.28			β-Ketoacyl-CoA synthase
VIT_06s0004g01250	2.16				Omega-6 fatty acid desaturase, endoplasmic reticulum (FAD2)
VIT_07s0005g01240	3.69	3.45	2.56	2.74	Triacylglycerol lipase
VIT_09s0002g04170		2.01			Acyl-CoA synthetases (Acyl-activating enzyme 13)
VIT_09s0002g06970	2.43				Palmitoyl-monogalactosyldiacylglycerol delta-7 desaturase, chloroplast
VIT_15s0046g02400		3.55	2.45		Glycerol-3-phosphate acyltransferase 8
VIT_18s0001g15460			3.61	3.42	Stearyl acyl carrier protein desaturase

Fig. 6. Overview of the major metabolic changes in response to *B. cinerea* infection in *V. vinifera* grapes at EL33 and EL35. Activation (+ shading in key) and inhibition (– shading in key) of the pathway as suggested by microarray data and increased content of metabolites at high and moderate levels are indicated. Ovals and hexagons represent metabolites and pathways, respectively. The expression profile of each individual gene and metabolite content is presented in [Supplementary Fig. S8A and B](#) for selected pathways. (This figure is available in colour at [JXB](#) online.)

Flavonoid biosynthesis was enriched at EL35 in infected berries (Fig. 5). Flavonoids, benzoic acids, and their derivatives have been indicated to play a role in plant defence (Graham, 1998; Wildermuth, 2006). Gallic acid or 3,4,5-trihydroxybenzoic acid and protocatechuic acid

or 3,4-dihydroxybenzoic acid, both with antifungal properties (Lattanzio *et al.*, 2006), were identified as positive markers of infection (Figs 3, 4, and 8), revealing the ability of infected fruits to initiate to some extent a defence response. Interestingly, gallic acid decreases during ripening

(Agudelo-Romero *et al.*, 2013b) so is a good candidate to be used as a marker of infection.

In combating pathogens, lignins are also synthesized to reinforce cell walls. Genes encoding enzymes involved in monolignol biosynthesis, including putative cinnamoyl-CoA reductases, increased their expression in *Arabidopsis* plants inoculated with *B. cinerea* (Ferrari *et al.*, 2007), as found in grapes at EL33 (VIT_02s0012g01570 and VIT_09s0070g00240; Fig. 6 and Supplementary Table S4).

However, as in unripe tomato fruit, *B. cinerea* induced the expression of genes also expressed during ripening of uninfected fruit. Among these at EL33 were genes encoding polygalacturonases (VIT_02s0025g00260) and expansins (VIT_00s1455g00010), which encode cell-wall-modifying proteins and have been shown to facilitate susceptibility (Cantu *et al.*, 2009).

We also noticed in infected berries upregulation of genes involved in the oxidative stress response (Fig. 5) such as those encoding glutathione *S*-transferase (VIT_19s0093g00390), glutaredoxin (VIT_06s0004g07040), and chitinase class IV (VIT_05s0094g00360), which play a well-known role in abiotic and biotic stress responses. In addition, increased content of the antioxidant α -tocopherol occurred in infected samples at EL33 and EL35 (Fig. 4A). However, the generation of oxidative burst that can trigger hypersensitive cell death is beneficial to necrotrophic pathogens such as *B. cinerea* that live in dead tissue. This fungus can exploit a host defence mechanism for its pathogenicity (Govrin and Levine, 2000). Plant cells produce reactive oxygen species after *B. cinerea* attack, which assist fungal colonization, since treatments with antioxidants suppress fungal infection (Asselbergh *et al.*, 2007).

A number of genes and metabolites induced in grapevine in response to *B. cinerea* are associated with the plant defence response. In particular, the phenylpropanoids are not synthesized by fungi, which suggests that *Botrytis*-susceptible Trincadeira is able to initiate to some extent a basal defence by induction of a defence-oriented reprogramming of the transcriptome and metabolome but is unable to restrict fungal growth or slow down disease progression. The same holds true for infection of grapevine leaves with powdery mildew resulting in expression of a substantial number of defence-related genes in a susceptible cultivar (Fung *et al.*, 2008; Polesani *et al.*, 2010; Perazzolli *et al.*, 2012). However, some of the defences that were established naturally in ripening grapes were suppressed in the infected grapes, which may determine the outcome of the fruit–pathogen interaction with benefits for the latter.

Biotic stress responses are putatively regulated by salicylic acid in healthy berries and by jasmonic acid and ethylene in infected berries

Generally, salicylic acid-mediated responses are associated with resistance to biotrophic pathogens, whereas jasmonate and ethylene mediate resistance to necrotrophic pathogens (Glazebrook, 2005).

In healthy berries, we observed an over-representation of genes involved in the salicylic acid-mediated signalling pathway in veraison-responsive transcripts (Supplementary

Table S5), namely those encoding *EDS1* homologous genes (Enhanced disease susceptibility 1, VIT_17s0000g07420 and VIT_17s0000g07370). The EDS1 protein along with its co-regulator Phytoalexin deficient4 (PAD4), is important in the regulation of the plant defence pathway involving salicylic acid signalling (Wiermer *et al.*, 2005). The gene encoding PAD4 (VIT_07s0031g02390) was even significantly downregulated at EL35 in infected berries when compared with veraison (although at a fold change of -1.55 ; Supplementary Table S4). Salicylic acid may be involved in the regulation of R-gene expression during activation of defence responses (Tan *et al.*, 2007) in healthy ripening grapes, a mechanism that seems to be absent in infected grapes.

Azelaic acid, which was identified as a positive marker of infection (Fig. 4B), has been reported as a component of plant systemic immunity. It is involved in priming the faster and stronger accumulation of salicylic acid in response to pathogen infection in *Arabidopsis* (Jung *et al.*, 2009). However, salicylic acid does not seem to be involved in the response to *Botrytis* attack. It may be that this pathogen activates the azelaic acid response, but the priming or another downstream process is suppressed or not recognized and therefore systemic acquired resistance and the salicylic acid response is not activated.

On the other hand, EDS1 and PAD4 have been indicated as negative regulators of ethylene/jasmonic acid defence signalling (Brodersen *et al.*, 2006). Our results suggested that the salicylic acid-mediated defence system is inhibited in infected berries, whereas the ethylene/jasmonic acid-mediated defence system is activated. In fact, mRNAs involved in the biosynthesis of jasmonic acid (VIT_18s0001g11630, VIT_03s0063g01820, and VIT_18s0041g02070; Supplementary Fig. S7) were upregulated at EL33 and some at EL35. The jasmonic acid-mediated signalling pathway was also enriched at EL33 in infected berries. Increased jasmonic acid synthesis may regulate the accumulation of resveratrol in infected grapes, as reported in grapevine cell cultures (Lijavetzky *et al.*, 2008). Genes involved in ethylene biosynthesis (VIT_12s0059g01380 and VIT_15s0046g02220) were also upregulated at EL33 and the expression of ethylene-responsive transcription factors was over-represented at EL 33 in infected berries (VIT_04s0008g03400 and VIT_16s0013g01110).

The results suggested that the response to *B. cinerea* attack in grapes is mediated by jasmonic acid and ethylene, as occurs in leaves of several plant species and in tomato (Blanco-Ulate *et al.*, 2013). In tomato, *B. cinerea* was shown to activate the synthesis of ethylene, thereby accelerating the onset of ripening and subsequently inducing susceptibility (Cantu *et al.*, 2009). In this work, we observed an acceleration of ripening in infected berries. In non-climacteric fruits such as grapes, this can be due to increased ethylene synthesis and, more likely, to increased content of abscisic acid (Fortes *et al.*, 2011), as suggested by the downregulation of a gene involved in its catabolism (VIT_18s0001g10500; Table 1 and Supplementary Fig. S8A).

However, unlike what is commonly found in the literature, particularly in tomato, susceptibility was already observed in green berries and did not seem to be directly dependent

on the onset of the ripening programme. Infection of green berries has also been observed in another Portuguese variety (Touriga Nacional), although the symptoms were less pronounced than in Trincadeira (data not shown). This fact questions previous assumptions that, between bloom and veraison, grape berries are resistant to infections occurring in nature (Goetz *et al.*, 1999). Although we observed very few natural infections at EL33 and EL35, the use of the term 'tolerance' instead of 'resistance' seems more appropriate. It should be noted, however, that when infections were performed at EL31/EL32, symptoms were no longer observed at EL33 and EL35 (data not shown). This age-related resistance may be due to the increased size and changes in the composition of the cuticle that no longer enabled the fungi to penetrate easily (Deytieu-Belleau *et al.*, 2009).

Many genes encoding transcription factors were upregulated in infected berries, such as a gene encoding WRKY DNA-binding protein 33 (VIT_06s0004g07500). The *Arabidopsis thaliana* transcription factor WRKY33 is essential for defence against the necrotrophic fungus *B. cinerea* (Birkenbihl *et al.*, 2012). The loss of WRKY33 function results in inappropriate activation of the salicylic acid-related host response and in the downregulation of jasmonic acid-associated responses. The upregulation of this gene in infected berries is therefore consistent with the putative activation of jasmonic acid-dependent processes, as mentioned previously. It is also possible that some of these transcription factors are involved in susceptibility towards pathogen attack. We observed the upregulation at EL33 of a gene encoding Myb domain protein 46 (VIT_13s0064g00960). In fact, *Arabidopsis myb46* mutants have enhanced disease resistance to *B. cinerea* (Ramirez *et al.*, 2011).

The increase in conjugated polyamines during grey mould infection of grapes has been described previously (Geny *et al.*, 2003), and these growth regulators are known to play a role in the biotic stress response. In fact, genes involved in polyamine biosynthesis are upregulated at EL33 and/or at EL35 (VIT_18s0001g00740 and VIT_05s0020g03200). Moreover, 4-aminobutanoic acid, or GABA, which is involved in polyamine metabolism (Agudelo-Romero *et al.*, 2013a), only appeared in infected grapes (Fig. 4). The accumulation of this compound in botrytized berries has been described previously (Hong *et al.*, 2012).

Auxins may also be involved in the response to *Botrytis* attack, as suggested by the enrichment in the functional class of auxin signalling (Fig. 5). Recently, an interaction between auxin and jasmonic acid was shown to occur in resistance to necrotrophic pathogens (Qi *et al.*, 2012).

Alterations in carbohydrate metabolism that either benefit the pathogen or are involved in defence

Gene expression related to carbohydrate metabolism is altered by the pathogen infection (Fig. 5), which manipulates it for its own need and often leads to a decrease in photosynthetic assimilate production (Berger *et al.*, 2007). We noticed downregulation of photosynthesis-related genes, in particular at EL35 in infected berries (VIT_00s0181g00180 and VIT_04s0044g01410; Fig. 6), as occurs in leaves of susceptible

grapevine cultivars upon powdery mildew infection (Fung *et al.*, 2008). Thus, it seems that infection by either biotrophic or necrotrophic pathogens shares common features in several tissues such as fruits and leaves.

We observed downregulation of a gene encoding Snf1 protein kinase (KIN10) (VIT_08s0040g00720) in infected samples (Supplementary Fig. S7 and Supplementary Table S4; fold change was -1.86 and -1.74 at EL33 and EL35, respectively). This kinase is inactivated by sugars (Baena-González *et al.*, 2007), which is in agreement with the increased content in fructose and glucose in infected samples (Fig. 2 and Supplementary Table S3). Elevated soluble carbohydrate concentrations suggest that a pathogen-induced carbohydrate sink is established. Although we cannot ascertain the origin of the sugars, it is likely that they are provided mainly from plant origin. In fact, uptake of plant hexoses by *B. cinerea* and their conversion to fungal metabolites was reported during the pathogenesis of sunflower (Dulermo *et al.*, 2009). Moreover, the amount of plant tissue was much higher than the number of cells from the colonizing fungus, as can be observed in Fig. 1 and Supplementary Fig. S1. The dehydration that occurs in *Botrytis*-infected grapes leading to a decrease in berry weight is also likely to play a role in the concentration of hexoses (Magyar, 2011). The detected increase in these metabolites putatively of fruit origin may partially be due to starch catabolism (as well as sugar import from leaves), as suggested by the upregulation of several genes encoding α -amylase (VIT_00s0270g00120 and VIT_17s0119g00280; Supplementary Table S4 and Supplementary Fig. S8). We cannot also rule out that some isoforms of invertase are active or that invertases from fungi are responsible for the increase in hexoses. Recently, sucrose was shown to decrease in grapes infected with *B. cinerea* (Hong *et al.*, 2012), suggesting that it is also contributing to the detected increase in glucose and fructose. The synthesis of this carbohydrate does not seem to be favoured, as indicated by the downregulation of a gene encoding sucrose-phosphate synthase (VIT_05s0029g01140; Table 1 and Supplementary Fig. S8). Alternatively, *B. cinerea* may prefer sucrose directly as a carbon source to glucose and fructose (Hong *et al.*, 2012).

Another striking feature was the enrichment in the functional classes of galactose and trehalose metabolisms (VIT_14s0060g00790, VIT_05s0077g00840, VIT_14s0066g00810, VIT_07s0005g01680, VIT_02s0154g00110, and VIT_17s0000g08010). Two of these enzymes are needed for raffinose synthesis, namely galactinol synthase and raffinose synthase. Raffinose is a minor carbohydrate in grape tissues, but it has been related to cold acclimation in *V. vinifera* (Grant *et al.*, 2009). It may be that accumulation of this osmoprotectant sugar also constitutes a response to biotic stress.

Our results suggested an inhibition of plastid glycolysis (VIT_03s0038g00670) and activation of cytoplasmic glycolysis (VIT_01s0010g02460; Supplementary Table S4 and Supplementary Fig. S8). Moreover, we observed a decrease in malic acid content at both EL33 and EL35 in infected samples along with the upregulation of a gene encoding malate dehydrogenase (VIT_19s0014g01640, fold change of 1.63 and 1.61 ,

respectively; [Supplementary Table S4](#) and [Supplementary Fig. S8](#)). Malate can be channelled to gluconeogenesis, as suggested by the upregulation of a gene encoding phosphoenolpyruvate carboxylase kinase (VIT_14s0036g00420, fold change of 1.51 at EL35) and to secondary metabolism ([Sweetman et al., 2009](#)). It should be taken into account that the metabolism of malate by the fungus may also play a role in this context, and further studies addressing the transcriptome of the fungus upon this interaction are necessary.

The glyoxylate cycle seemed to be activated, as suggested by the upregulation of genes encoding ATP-citrate synthase, glyoxysomal malate synthase, and isocitrate lyase (VIT_01s0127g00260, VIT_17s0000g01820, and VIT_12s0059g02350). This indicates that fatty acid degradation (β -oxidation) is probably activated.

Gluconic acid was hardly detected in control berries. This compound of mainly fungal origin has been suggested as an indicator of rotten grapes ([Crachereau, 2004](#)) and has been found in botrytized berries ([Hong et al., 2012](#)). On the other hand, *myo*-inositol, arabitol, and ribitol ([Fig. 4](#)) can be used together with 1-*O*-methyl- glucopyranoside as biomarkers of an early stage of infection.

Overall, *B. cinerea* induced a reprogramming of carbohydrate metabolism in the grapes with a decrease in photosynthesis and increase in catabolism of starch and malate with putative activation of gluconeogenesis and the glyoxylate cycle to provide more sugars that might be metabolized by the fungus or to provide precursors of plant secondary metabolites involved in defence ([Fig. 6](#)).

Alterations in lipid metabolism are putatively linked to jasmonic acid and defence

Alterations in lipid metabolism gene expression occur upon *Botrytis* infection as suggested by the enrichment in the classes of α -linolenic acid metabolism, glycerolipid catabolism, and sphingolipid metabolism ([Fig. 5](#)). Moreover, we observed significant changes in the contents of glycerol and fatty acids such as 9-octadecenoic acid (Z), or oleic acid, which is a biomarker of infection ([Fig. 4](#)). Although we cannot be sure of the origin of the measured fatty acids, microarray results suggested that fatty acids are synthesized in the infected fruits. Moreover, fatty acids present significant antifungal activities including against *B. cinerea* ([Walters et al., 2004](#); [Calvo-Garrido et al., 2014](#)) so it is unlikely that they are produced by the fungus upon interaction with the grapes. In addition, the amount of plant tissue analysed was much higher than the corresponding fungal tissue, as mentioned previously. A gene encoding a stearyl acyl carrier protein desaturase (VIT_18s0001g15460), which catalyses the desaturation of stearic acid to oleic acid, was upregulated in infected berries at EL33 and EL35 (fold change of 1.71 and 1.80, respectively; [Supplementary Table S4](#)) and may be involved in the detected increase in oleic acid. This fatty acid can eventually enter glycerolipid synthesis, as suggested by the upregulation of related genes (VIT_15s0046g02400 and VIT_13s0019g04730).

It has been shown that a reduction in oleic acid levels results in constitutive activation of the salicylic acid-dependent pathway and repression of the jasmonic acid-dependent

pathway ([Kachroo et al., 2003](#)). Our results support these data, since increased oleic acid levels seemed to correlate with jasmonic acid signalling in infected berries. In fact, oleic acid levels regulated by glycerolipid metabolism modulate defence gene expression in *Arabidopsis* ([Kachroo et al., 2004](#)).

Hexadecanoic, octacosanoic, tetracosanoic, and triacontanoic acids increased in infected berries at EL33 and EL35 ([Figs 3 and 4](#)). Hexadecanoic acid (palmitic acid) has been suggested as a biomarker of resistance of grapevine against downy mildew ([Batovska et al., 2009](#)) and is involved in the impermeability of cell lipid membranes ([Agafonov et al., 2003](#)), hampering fungal infiltration. On the other hand, saturated long-chain fatty acids are major constituents of grape waxes that accumulate in ripe berries. This may imply that acceleration of ripening is occurring in grapes due to *Botrytis* infection, as occurs in tomato ([Cantu et al., 2009](#)). Fatty acids can be activated by acyl-CoA synthetases (VIT_03s0063g01880 and VIT_09s0002g04170). The resultant acyl-CoAs can be used for wax and cutin production, which are components of berry skins and prevent dehydration.

Heptadecanoic acid, 9,12-octadecadienoic acid (Z,Z) (linoleic acid), 9,12,15-octadecatrienoic acid (Z,Z,Z) (α -linolenic acid), 9-octadecenoic acid (Z), and tricosanoic acid only appeared in infected berries, at both EL33 and EL35 ([Fig. 4](#)).

A gene encoding a chloroplastidial omega 6-fatty acid desaturase (FAD6, VIT_06s0004g01250) was upregulated at EL33 in infected berries. This enzyme is the primary route of polyunsaturated fatty acids production in plants and converts oleic acid to linoleic acid. In fact, many genes involved in fatty acid biosynthesis were upregulated at EL33 or EL35 in infected berries (VIT_09s0002g06970, VIT_01s0011g04380, and VIT_04s0008g02250). Our results therefore suggested increased synthesis of fatty acids and glycerolipids in response to *Botrytis* infection. However, some of these fatty acids may be degraded, as stated previously. A gene encoding an acyl-CoA oxidase (VIT_00s0662g00010) putatively involved in β -oxidation of fatty acids (glyoxylate cycle) was upregulated at EL33 ([Fig. 6](#); fold change of 1.66). This would provide the grape cells with extra sugars and precursors for the synthesis of secondary metabolites. Triacylglycerol lipases (VIT_07s0005g01240) may be involved in the release of fatty acids for this purpose. The degradation of α -linolenic acid may be partially directed to the synthesis of jasmonic acid, which seems to be activated in infected berries as described above.

Conclusions

This work provides the first combined transcriptomic and metabolomic analysis of the response of a fleshy fruit against a pathogen. The initiation of defence mechanisms against a pathogen is often reported for vegetative tissues with organs like the fruits being neglected. Furthermore, we provide evidence that infection may occur in green fruits against what is currently found in the literature if the appropriate conditions are given and the cultivar is extremely susceptible. Thus, the infection does not necessarily depend upon the initiation of

the ripening programme, and green fruits are likely to exhibit tolerance against fungal infection instead of resistance, as frequently mentioned. However, acceleration of ripening, in particular at veraison, occurs with the infection, as previously shown for tomato (Cantu *et al.* 2009).

We observed a profound change in the transcriptome and metabolome of the berries towards the defence response with the putative involvement of jasmonic acid and ethylene. The reprogramming of primary metabolism seems to have two major goals, providing extra precursors for the synthesis of secondary metabolites that play a role in plant defence and providing the pathogen with carbon sources. However, there are defences that are established naturally in ripening berries that are absent in infected berries, highlighting the importance of conducting studies in these organs. The strategy of grapevine improvement through conventional breeding or genetic engineering should therefore aim at avoiding the shut-down caused by the pathogen of particular defences, namely those involving salicylic acid that regulate R genes and are activated within the normal ripening programme.

Finally, this study provides biomarkers such as gallic and azelaic acids, arabitrol, ribitol, 4-amino butanoic acid, 1-*O*-methyl- glucopyranoside, and several fatty acids that can be tested for the monitoring of infection of grapes at the early infection stage in the vineyard, thus enabling a more appropriate management strategy and more controlled use of fungicides that pose an environmental and health impact.

Supplementary data

Supplementary data are available at *JXB* online.

Supplementary Table S1. List of primers used in real-time reverse transcription-PCR.

Supplementary Table S2. Evaluation of symptoms at EL33 following infection with *Botrytis cinerea* at EL29.

Supplementary Table S3. Normalized responses of profiled soluble and volatile metabolites from developing berries of *Vitis vinifera* cv. Trincadeira.

Supplementary Table S4. List of the 9169 genes differentially expressed in infected berries at EL33 and EL35 and also in mock-treated berries at veraison (EL35).

Supplementary Table S5. Functional enrichment analysis of transcripts differentially expressed in infected Trincadeira berries and at veraison.

Supplementary Fig. S1. Cluster of Trincadeira grapes at EL31 infected with *Botrytis cinerea*.

Supplementary Fig. S2. Evaluation of infection of biological replicates at EL33 and EL35.

Supplementary Fig. S3. Analysis of infection-responsive metabolites from developing berries of *Vitis vinifera* cv. Trincadeira.

Supplementary Fig. S4. Analysis of veraison-responsive metabolites from developing berries of *Vitis vinifera* cv. Trincadeira.

Supplementary Fig. S5. The changes of organic acid metabolism in developing berries of *Vitis vinifera* cv. Trincadeira at developmental stage EL33 and EL35 (veraison) after *Botrytis cinerea* infection and in non-infected berries.

Supplementary Fig. S6. PCA plot showing transcriptional discrimination of developmental stages and infected vs control berries according to their RMA normalized expression data.

Supplementary Fig. S7. Real-time qPCR validation of the expression profiles of six genes upon *Botrytis cinerea* infection of Trincadeira cultivar berries at two stages of fruit development (pre-veraison: EL33, and veraison: EL35).

Supplementary Fig. S8. Transcripts and metabolites showing differences between infected and control berries (EL33-A and EL35-B); pathways are represented in VitisNet networks (Grimplet *et al.*, 2009) with the Cytoscape software.

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