A transcriptomic study of grapevine (Vitis vinifera cv. Cabernet-Sauvignon) interaction with the vascular ascomycete fungus Eutypa lata

Céline Camps¹, Christian Kappel¹, Pascal Lecomte², Céline Léon¹, Eric Gomès¹, Pierre Coutos-Thévenot³ and Serge Delrot¹,*

¹ Institute of Vine and Wine Sciences, UMR 1287 Ecophysiologie and Grape Functional Genomics, University of Bordeaux, INRA, 210 Chemin de Leysotte, CS 50008, 33882 Villenave d’Omon, France
² Institute of Vine and Wine Sciences, UMR Santé Végétale, INRA-ENITA, BP81-33883 Villenave d’Omon, France
³ FRE CNRS 3091, University of Poitiers, 40 Avenue du Recteur Pineau, France

* To whom correspondence should be addressed. E-mail: serge.delrot@bordeaux.inra.fr

Received 25 November 2009; Revised 24 January 2010; Accepted 2 February 2010

Abstract

Eutypa dieback is a vascular disease that may severely affect vineyards throughout the world. In the present work, microarrays were made in order (i) to improve our knowledge of grapevine (Vitis vinifera cv. Cabernet-Sauvignon) responses to Eutypa lata, the causal agent of Eutypa dieback; and (ii) to identify genes that may prevent symptom development. Qiagen/Operon grapevine microarrays comprising 14 500 probes were used to compare, under three experimental conditions (in vitro, in the greenhouse, and in the vineyard), foliar material of infected symptomatic plants (S+R+), infected asymptomatic plants (S –R+), and healthy plants (S –R–). These plants were characterized by symptom notation after natural (vineyard) or experimental (in vitro and greenhouse) infection, re-isolation of the fungus located in the lignified parts, and the formal identification of E. lata mycelium by PCR. Semi-quantitative real-time PCR experiments were run to confirm the expression of some genes of interest in response to E. lata. Their expression profiles were also studied in response to other grapevine pathogens (Erysiphe necator, Plasmopara viticola, and Botrytis cinerea). (i) Five functional categories of genes, that is those involved in metabolism, defence reactions, interaction with the environment, transport, and transcription, were up-regulated in S+R+ plants compared with S–R– plants. These genes, which cannot prevent infection and symptom development, are not specific since they were also up-regulated after infection by powdery mildew, downy mildew, and black rot. (ii) Most of the genes that may prevent symptom development are associated with the light phase of photosynthesis. This finding is discussed in the context of previous data on the mode of action of eutypin and the polypeptide fraction secreted by Eutypa.

Key words: Eutypa dieback, Eutypa lata, grapevine, microarrays, transcriptome, Vitis vinifera.

Introduction

Eutypa dieback is a wood decay disease found in all grape-growing areas, which can be very damaging (Munkvold et al., 1994; Wicks et al., 1999; Creaser et al., 2001). Eutypa dieback is caused by the vascular ascomycete fungus Eutypa lata (Moller and Kasimatis, 1978). After initial infection by the fungus, a lag phase of several years is often observed before the appearance of symptoms (Duthie et al., 1991; Tey-Ruhl et al., 1991) whose intensity on a given plant may vary with each year (Creaser et al., 2001). Symptoms of Eutypa dieback include stunting of growing shoots after bud break, with small, cupped, chlorotic, and tattered leaves, reduced development of fruit clusters, and characteristic dark, wedge-shaped necrosis of the trunk and cordons (Lecomte et al., 2000; Mahoney et al., 2003). Leaf
symptoms are due both to toxins (Mauro et al., 1988; Tey Rulh et al., 1991; Deswarte et al., 1996; Molyneux et al., 2002; Mahoney et al., 2003; Smith et al., 2003) and to cell wall-degrading enzymes (English and Davis, 1978; Elghazali et al., 1992; Schmidt et al., 1999; Rolshausen et al., 2008) produced by the fungus in the wood (Bernard and Mur, 1986). Variations of disease expression may also depend on cultivar susceptibility (Péros and Berger, 1994; Sosnowski et al., 2007). Among the most cultivated grapevine cultivars, Cabernet-Sauvignon is particularly susceptible to Eutypa dieback (Péros and Berger, 1994). There is no known resistant cultivar (Boubals, 1986; Mauro et al., 1988; Munkvold and Marois, 1995; Péros and Berger, 1994; Chapuis et al., 1998; Sosnowski et al., 2007), and neither efficient treatment nor non-destructive diagnostic tools are available for this disease. Thus, in cases of contamination, infected plants die within a few years (Pascoe, 1999). Finally, except for some microscopic and toxicological studies (Philippe et al., 1993; Deswarthes et al., 1994, 1996; Amborabé et al., 2001; Kim et al., 2004; Octave et al., 2006b), grapevine responses to E. lata are still poorly described.

The present work describes a transcriptomic study of grapevine (Vitis vinifera cv. Cabernet-Sauvignon) response after infection by the vascular ascomycete fungus E. lata. The aims of this work are to (i) characterize grapevine responses to E. lata infection and (ii) to identify genes more specifically associated with a lack of symptoms. For these purposes, leaves of infected symptomatic plants (S+R+), infected asymptomatic plants (S,R+), and healthy plants (S,R-) from vineyard (natural infection), greenhouse (experimental infection), and in vitro (experimental infection) material were compared.

**Materials and methods**

**Infection and sampling**

Two conditions were used for the production of infected and healthy Cabernet-Sauvignon grapevines: the vineyard (natural infection) and the greenhouse (experimental infection).

Vineyard samples were collected in an INRA experimental plot (Chateau Cruzeaux) located close to Bordeaux. In this vineyard, which is naturally infected by E. lata, Eutypa dieback symptoms were monitored every year between 2002 and 2006. Healthy grapevines were selected among those that did not show disease symptoms during this time. Infected grapevines showing apparent Eutypa dieback symptoms every year from 2002 to 2006 were also selected. Leaf samples were collected in June when symptoms were most visible, immediately frozen in liquid nitrogen, and stored at −80°C. Absence of infection by other fungal pathogens (Botrytis cinerea, Erysiphe necator, and Plasmopora viticola) was visually checked during sampling.

Two-node Cabernet-Sauvignon cuttings were rooted 2 months before infection and grown in a greenhouse (Chapuis, 1995). The temperature was maintained between 20°C and 32°C. Plants were watered for 5 min, twice per day, using 0.5 l h⁻¹ emitters via a drip system. They received, on average, 18 h of light per day from both ambient and supplemental lighting. These rooted cuttings were experimentally infected with the E. lata strain BX1-10, which has been characterized as a very aggressive strain (Péros and Berger, 1999). Infections were carried out as described by Chapuis (1995). A hole (2 mm diameter, 5 mm deep) was drilled 2 cm below the upper bud. After 10–15 d of culture at 23°C in darkness, E. lata mycelium was collected by scraping the surface of the PDA (potato dextrose agar, Difco) culture medium with a scalpel, and suspended in sterile water with strong agitation. A 20 µl aliquot of this suspension was injected into the hole in the cutting and the inoculation site was immediately covered with paraffin. Non-inoculated control vines treated with 20 µl of sterile water were included in the experiment. Cuttings were maintained in the greenhouse until eutypiosis symptoms appeared the following year. An average of 10 leaves were randomly collected from each grapevine, immediately frozen in liquid nitrogen, and stored at −80°C. All samples were collected at the same time.

**Notation of leaf Eutypa dieback symptoms**

In the vineyard, Eutypa symptoms were followed between 2002 and 2006 according to the guidelines provided by Darrieutort and Lecomte (2007). In the greenhouse, leaf symptoms were evaluated for each cutting 1 year after the experimental infection and categorized as not visible (S) or visible symptoms (S+) (for severe, moderate, or mild symptoms), as suggested by Péros and Berger (1994).

**Recovery of the fungus**

For both vineyard and greenhouse plants, cross-sections were made in woody parts to look for brown lesions characteristic of Eutypa dieback as described by Lecomte et al. (2000). After surface sterilization by rapid flaming, a wood fragment was sampled along the margin of the lesion (between healthy and infected wood), using pruning shears. This segment was then split into wood chips (3 × 5 × 5 mm) for culture of E. lata. Chips were surface sterilized by soaking in 3% calcium hypochlorite solution. They were placed in sterile conditions onto Petri dishes containing malt (15 g l⁻¹), agar (20 g l⁻¹) medium supplemented with chloramphenicol (50 mg l⁻¹). Petri plates with both greenhouse and vineyard samples were assessed visually for the presence of E. lata, after 10 d of incubation in the dark at 22°C. When the samples were for positive E. lata, a white cottony mycelium growth originating from the sample was observed.

**Identification of E. lata by PCR**

PCR identification of E. lata was carried out as described previously (Lardner et al., 2005). After rapid DNA extraction from re-isolated mycelium, amplification was performed using the SCAR primer pair EuTo2 F3 (TGGTGGACGGGTAGGT TAG) and EuTo2 R2 (GGCCTTACGAAATAGACCAA). This indirect and destructive PCR allowed a clear identification of the presence of E. lata in infected plants. Rapid DNA extraction from the mycelium was carried out according to Hamelin et al. (2000). Briefly, a small amount of mycelium was removed from the surface of actively growing cultures on PDA using a 200 µl pipette tip, incubated for 7 min at 95°C in 100 µl of extraction buffer (0.5 M TRIS-HCl, pH 9. 0.1% Triton X-100), then cooled on ice for 5 min. PCRs were conducted with 1 µl aliquots of fungal DNA extract (~30 ng of template) in a total volume of 25 µl. Each reaction also contained 0.2 vol. of 5x green buffer (Promega), 2 mM MgCl₂, 200 µM each of dATP, dCTP, dGTP, and dTTP (Roche diagnosis), 0.2 µM of each primer (Operon technologies), and 1 U of GoTaq DNA polymerase (Promega). An initial denaturation step of 2 min at 94°C was followed by 37 cycles of 30 s at 94°C, 30 s at 58°C, and 1 min at 72°C, with a final extension of 10 min at 72°C. Before migration, 0.2 vol. of loading buffer (30% glycerol, 0.25% bromophenol blue, 0.25% xylene cyanol) was added to the samples. Amplification products, which have an expected size of 643 bp, were separated by electrophoresis in 2% agarose gels using a 0.5x TAE buffer (20 mM TRIS-HCl, 0.5 mM EDTA, 2.5 mM Na acetate), stained with 100 µg l⁻¹ ethidium bromide (Biorad), and visualized under UV illumination ‘GEL DOC 2000’ (Biorad).
Eutypa lata isolation and PCR enabled the determination of whether the non-inoculated control or the selected grapevines that seemed to be healthy were indeed axenic (negative isolation), and to separate the experimentally inoculated samples that became infected (positive recovery and PCR test) from those that did not (negative re-isolation). R⁺ samples correspond to positive recovery and positive PCR, whereas samples were rated R⁻ in the case of negative isolation.

Infection of detached leaves with P. viticola, E. necator, and B. cinerea
In order to determine whether key changes in gene expression in leaves infected with E. lata (identified by transcriptomic studies) were specific to this pathogen, they were also profiled by real-time PCR (RT-PCR) in vine leaves infected with other fungal pathogens.

Plasmopara viticola: Healthy leaves were sampled just before infection from Cabernet-Sauvignon vines grown in the greenhouse. They were placed upper face down in a Petri dish. Half of them were infected with 15 μl droplets of a P. viticola spore suspension (3000 spores ml⁻¹, counted with a Malassez cell) deposited on the lower face of the leaf; the other half were left as the non-infected control. The leaves were maintained in a growth chamber at 22 °C under a photoperiod of 16 h light/8 h darkness. Leaves infected with various strains of P. viticola (PAV 32, FEM 03, PIC 59, MIC 128, EAU 14, and FET 03) were collected 12, 14, and 16 d after infection. At each time of infection, leaves infected by these different strains were pooled together. Healthy leaves were also collected after 12, 14, and 16 d in a Petri dish. These samples were deep-frozen in liquid nitrogen and used later for RT-PCR studies on candidate genes.

Erysiphe necator: Mature leaves from Cabernet-Sauvignon vines grown in the greenhouse were collected and, after sterilization in calcium hypochlorite (50 g l⁻¹) for 10 min, they were placed in a Petri dish containing solid medium (15 g l⁻¹ agar, with 30 mg l⁻¹ benzimidazole, upper face upwards). The fungal conidia were detached from a pre-inoculated sporulating leaf by an air stream, and inoculated by gravity under dry conditions on the selected leaves.

Botrytis cinerea: Chardonnay grapevine plantlets grown in vitro on MacCown medium were transferred to aeroponic conditions when the fourth leaf was developing and the roots were 4–5 cm long. The plantlets were placed in a container where the nutrient solution was sprayed as a mist. The container was maintained in a growth cabinet under a sodium bulb, with constant temperature (23 °C) and humidity (75%). The 916 T. B. cinerea strain was grown on malt agar (10 g l⁻¹; 15 g l⁻¹) and induced to sporulate by continuous light for 5–10 d. A conidial suspension was prepared with sterile distilled water and maintained on ice until inoculation. Infection was carried out by deposition of 8.5 μl (~1000 conidia) of this suspension onto the leaf. Several healthy leaves (0 h) or infected leaves were collected 24, 48, and 72 h after infection.

RNA isolation and labelling
RNA isolation was carried out as described previously by Reid et al. (2006). To prepare the fluorescent targets, total RNA was amplified using the Amino Allyl MessageAmp II aRNA Amplification Kit (Ambion, TX, USA) following the manufacturer’s instructions. The first-strand cDNA was synthesized from 2 μg of total RNA with ArrayScript and T7 oligo(dt) primer, after incubation for 2 h at 42 °C. The cDNA then underwent second-strand synthesis (2 h at 16 °C) and was cleaned-up with the same kit to become a template for in vitro transcription with T7 RNA polymerase. During transcription (14 h at 37 °C) a modified nucleotide, amino allyl UTP, is incorporated into the aRNA. Amino allyl UTP contains a reactive primary amino group that can be chemically coupled to NHS ester dyes. A 25 μg aliquot of amino allyl aRNA was used for this subsequent indirect labelling with the fluorescent cyanine dyes Cy3-dCTP and Cy5-dCTP (Amersham Biosciences, USA).

Microarray experiments
In order to characterize grapevine response to E. lata infection, gene expression was profiled in infected plants with symptoms (S⁻R⁻), infected plants without symptoms (S⁺R⁺), and healthy plants (S⁻R⁺) produced in two experimental conditions: the greenhouse and the vineyard. Fluorescent targets prepared with RNA extracted from leaves of these plants (S⁻R⁻, S⁺R⁺, and S⁻R⁺) were hybridized to 70mer oligonucleotide microarrays, allowing simultaneous monitoring of the expression of ~15 000 grapevine genes. Microarrays were used to perform three different comparisons (Fig. 4): for the first comparison (S⁻R⁻/S⁺R⁺), three biological replicates were used in vineyard condition and two biological replicates were used for the greenhouse material. For the second comparison (S⁻R⁻/S⁻R⁺), three and two biological replicates were used, respectively, in greenhouse and vineyard conditions. For the last comparison (S⁻R⁻/S⁺R⁺), two biological replicates were made in the greenhouse condition and one biological replicate was made in the vineyard condition. At least two technical replicates (dye swap) were made for each comparison. The data are available in ArrayExpress (http://www.ebi.ac.uk/arrayexpress) under the accession number E-MEXP-2337.

Greenhouse and vineyard microarray data were combined with microarray data that we obtained previously with in vitro plantlets experimentally infected by E. lata, and that were used to test the Mapman software presently being adapted for grapevine (Rotter et al., 2009). These in vitro microarray data can be found under the accession number E-MEXP-2102 in Array Express.

Hybridization
For microarray production, the Array-Ready Oligo Set™ for the grape (V. vinifera) genome Version 1.0 designed by Operon was used. This set contains 14 562 probes of 70mer representing 14 562 transcripts from The Institute for Genomic Research (TIGR) Grape Gene Index (VvGI), release 3. Oligonucleotide probes were mapped to the grapevine genome (Jailion et al., 2007) and to the most recent release of the DFCI Grape Gene Index (version 6.0). Genome transcripts have been annotated automatically against the Swissprot database. Manual annotation has been done for differentially expressed genes using Uniprot’s Uniref100 database. Probes were synthesized by Qiagen and spotted onto epoxy mirror slides (Amersham) at the Montpellier Languedoc Roussillon Genopole, Institut de Génomique Fonctionnelle, at a concentration of 5 μm and a spot size of 150–160 μm. Just before hybridization, oligonucleotides were fixed onto the slide by UV (254 nm) radiation of 120 mJ in a UV Stratalinker 2400-cross-linker (Stratagene, USA). The slides were then washed with up and down gentle movement, twice in 0.2% SDS for 1 min and twice in distilled water for 5 min. Air-dried slides were positioned in the hybridization chambers.

For each hybridization, 600 pmol (~4 μg) of Cy3 and Cy5 aRNA targets were mixed. Fragmentation was carried out for 15 min at 70 °C with an RNA fragmentation reagent kit (Ambion). The final volume of the target solution was then adjusted to 100 μl with hybridization solution; 50% formamide, 5× Denhardt’s solution, 1× SSC, 0.05% SDS, and 1 μg ml⁻¹ denatured salmon sperm DNA (Stratagene, USA). This target solution was finally denatured for 2 min at 95 °C, cooled on ice for 2 min, and stabilized at 37 °C until injection (maximum 5 min). During injection, denatured target solution (600 pi of Cy3- and Cy5-labelled aRNA) was introduced into the hybridization chamber containing the microarrays slide (14 562 grapevine oligo probes). Hybridization was then conducted for 16 h at 37 °C, with moderate agitation, in the automated microarray station HS4800 Mastersystem (Tecan). Slides were washed sequentially at 30 °C in 1× SSC/0.2% SDS for 20 min in 0.1× SSC/0.2% SDS for
10 min, twice; and finally in 0.1× SSC for 10 min. The washed arrays were quickly dried with 2.7 bars of nitrogen gas and immediately scanned.

Microarray data analysis

The microarrays were scanned with a GenePix 4000B fluorescence reader (Axon Instruments, Canada) using GenePix 4.0 image acquisition software. It simultaneously scans array slides at two wavelengths using a dual-laser scanning system. These wavelengths (352 nm and 635 nm) are used to excite the fluorophores Cy3 and Cy5, respectively. A pair of photomultiplier tubes (PMTs) is used to detect the emitted fluorescent light. Sensitivity of detection can be adjusted by changing the voltage applied to the PMT. PMT voltages were adjusted to 400 V for Cy3 (352 nm) and 460 V for Cy5 (635 nm) in order to obtain maximal signal intensities and low saturation <1%.

The microarray images obtained with the GenePix 4000B scanner were quantified with the Maia tool version 2.75 (Novikov and Barillot, 2007). A full version of the software is freely available to non-commercial users upon request from the authors. Maia 2.75 allowed an automatic processing of the two-colour microarray images including: localization of spots with different morphological characters, quantification, and quality control. Flagged and saturated (intensity >50 000) spots were filtered out and excluded from further analysis.

Array normalization was carried out using a modified version of the Goulphar script version 1.1.2 (Lemoine et al., 2006) to take into account input data in the MAIA format. Median intensity data without background subtraction were normalized by a global lowess method followed by a print-tip median method. The lowess function enables the correction of global intensity artefacts due to the difference in incorporation between the two dyes. The print-tip method allows the correction of the spatial intensity artefacts due to the print-tips.

Differentially expressed genes were identified with the R/Bioconductor package Limma (Smyth, 2004, 2005) using linear models and by taking into account technical and biological replicates. Genes with a P-value ≤0.05 and an expression ratio ≥1.4 were deemed potentially significant and selected for further study. For convenience and clarity of the text, although what was actually measured were transcript amounts, and not transcriptional activities, reference is made to ‘up’- or ‘down-regulation’, and to ‘over-’ and ‘underexpression’.

RT-PCR expression profiles of candidate genes

The expression profiles of candidate genes were studied by semi-quantitative RT-PCR in response to E. lata and other grapevine pathogens (E. necator, P. viticola, and B. cinerea).

TC sequences (Grape Gene Index Version 6) or grapevine predicted gene genomic sequences (Jaillon et al., 2007), revealing 100% homology to the microarray 70mer oligonucleotides, were used to design gene-specific primers located in the 3' untranslated region and in the penultimate exon with Primer 3 and NetPrimer software. These primers were then synthesized by Operon. Primer sequences and predicted product size are given in Supplementary Table S1 available at JXB online.

About 100 ng of total RNA were reverse transcribed in a total volume of 25 μl with M-MLV reverse transcriptase (Promega). RNA was mixed with 3 μl of 10 μM oligo(dT), and adjusted to a final volume of 15 μl. The mixture was incubated at 75 °C for 10 min and snap-cooled on ice. The following preparation (10 μl) was then added to the RNA mixture: 5 μl of M-MLV reverse transcriptase reaction buffer (5×; Promega), 2 μl of deoxynucleoside triphosphate (10 mM each) mix, 1 μl of dithiothreitol (DTT; 100 mM), 1 μl of RNasin RNase inhibitor (40 U μl⁻¹; Promega), and 1 μl of M-MLV reverse transcriptase (200 U μl⁻¹; Promega). Incubation was at 42 °C for 1 h and final denaturation at 100 °C for 5 min. The cDNA solution was diluted with 100 μl of water. PCRs were conducted in triplicate in a total volume of 25 μl containing: 2.5 μl of diluted cDNA solution, 12.5 μl of GoTaq Green Master Mix 2X (Promega), and 1.25 μl of each primer (10 μM). GoTaq Green Master Mix (Promega) is a pre-mixed ready-to-use solution containing Taq DNA polymerase, dNTPs, MgCl₂, and reaction buffers at optimal concentrations for efficient amplification of DNA templates by PCR. DNA amplification was performed on a programmable thermal cycler (Progene, Techne, Cambridge, UK) with the following parameters: 95 °C for 5 min followed by 25-30 cycles of 95 °C for 30 s, 30 s at the specific primer pair annealing temperature, and 72 °C for 45 s, with a final cycle at 72 °C for 5 min.

Results

Characterization of plant material

Greenhouse conditions: One hundred and fifty Cabernet-Sauvignon cuttings grown in greenhouse conditions were infected through a stem drill with the BX1-10 E. lata strain. Control cuttings were maintained under the same greenhouse conditions. One year after infection, the symptoms were evaluated and ranked as severe, moderate, mild, or absent (Fig. 1). Among the 150 infected plants, 50% showed symptoms. Thirty-two cuttings exhibited severe symptoms,
21 cuttings showed moderate symptoms, and mild symptoms were found on seven plants. None of the 20 control plants showed symptoms. *Eutypa lata* recovery tests were conducted on 15 infected cuttings showing symptoms (five with severe, five with moderate, and five with mild symptoms), on 20 infected cuttings which did not develop symptoms, and on 10 control cuttings (Table 1). For the reisolation of fungal hyphae, the cutting was split longitudinally, and the zone adjacent to the necrosis was cut into 20 small pieces that were briefly surface-sterilized in a 3% sodium hypochlorite solution. These pieces were then placed onto culture medium. *Eutypa lata* was successfully re-isolated from all the infected plants showing symptoms, whereas no fungal growth was observed for nine out of 10 uninfected plants. *Eutypa lata* was also successfully re-isolated from most of the infected plants that did not show

Table 1. Results of fungal isolations from greenhouse cuttings experimentally infected with the BX1-10 *E. lata* strain

| Type of plant | Cutting no. | Symptoms | *E. lata* (rate/20) | Botryosphaeria | Penicillium | Aspergillus | Epicoccum |
|---------------|-------------|----------|---------------------|----------------|-------------|-------------|-----------|
| Infected      | 1           | Severe   | 20                  | –              | –           | –           | –         |
|               | 2           | Severe   | 12                  | –              | +           | –           | +         |
|               | 3           | Severe   | 12                  | –              | +           | +           | –         |
|               | 4           | Severe   | 9                   | +              | –           | –           | –         |
|               | 5           | Severe   | 6                   | +              | +           | –           | –         |
|               | 6           | Moderate | 13                  | +              | –           | –           | +         |
|               | 7           | Moderate | 11                  | –              | +           | –           | –         |
|               | 8           | Moderate | 6                   | +              | +           | +           | –         |
|               | 9           | Moderate | 5                   | +              | –           | –           | –         |
|               | 10          | Moderate | 5                   | +              | –           | –           | –         |
|               | 11          | Mild     | 13                  | –              | –           | –           | +         |
|               | 12          | Mild     | 11                  | –              | +           | –           | –         |
|               | 13          | Mild     | 9                   | +              | +           | –           | –         |
|               | 14          | Mild     | 5                   | +              | –           | –           | –         |
|               | 15          | Mild     | 4                   | +              | –           | –           | –         |
|               | 16          | None     | 15                  | +              | –           | –           | +         |
|               | 17          | None     | 13                  | –              | +           | –           | –         |
|               | 18          | None     | 12                  | –              | +           | –           | –         |
|               | 19          | None     | 10                  | +              | –           | –           | –         |
|               | 20          | None     | 10                  | +              | +           | –           | –         |
|               | 21          | None     | 10                  | –              | –           | –           | –         |
|               | 22          | None     | 10                  | +              | –           | –           | –         |
|               | 23          | None     | 9                   | –              | +           | +           | –         |
|               | 24          | None     | 6                   | +              | +           | –           | –         |
|               | 25          | None     | 6                   | –              | +           | –           | –         |
|               | 26          | None     | 5                   | +              | –           | –           | –         |
|               | 27          | None     | 5                   | –              | +           | –           | –         |
|               | 28          | None     | 4                   | –              | +           | –           | –         |
|               | 29          | None     | 4                   | –              | +           | –           | –         |
|               | 30          | None     | 4                   | –              | –           | +           | +         |
|               | 31          | None     | 3                   | +              | +           | –           | –         |
|               | 32          | None     | 2                   | +              | +           | –           | –         |
|               | 33          | None     | 1                   | –              | –           | +           | –         |
|               | 34          | None     | 0                   | +              | –           | –           | –         |
|               | 35          | None     | 0                   | +              | +           | –           | –         |
| Uninfected    | 1           | None     | 0                   | +              | +           | –           | +         |
|               | 2           | None     | 0                   | –              | –           | –           | –         |
|               | 3           | None     | 0                   | –              | +           | –           | –         |
|               | 4           | None     | 0                   | +              | –           | +           | –         |
|               | 5           | None     | 0                   | –              | –           | –           | –         |
|               | 6           | None     | 0                   | –              | +           | –           | –         |
|               | 7           | None     | 0                   | +              | –           | –           | –         |
|               | 8           | None     | 0                   | –              | –           | –           | +         |
|               | 9           | None     | 0                   | +              | +           | –           | –         |
|               | 10          | None     | 1                   | +              | –           | –           | –         |

Thirty-five plants exhibiting various degrees of symptoms were compared with 10 uninfected plants. Bold (S–R+), italics (S–R–), and bold italics (S+R–) identify plants that were selected for microarray analysis.
any symptoms (Table 1). The nine control plants that did not show any fungal growth and the infected plants for which at least nine fragments out of 20 gave a positive re-isolation result were selected for further analysis (Table 1).

**Vineyard plants: Eutypa dieback symptoms were studied** every year between 2002 and 2006 in the Châteaux Cruzeaux vineyard (Table 2). This allowed identification of 12 plants which showed symptoms of varying severity every year and 15 plants which did not show any symptoms during this period. The infected plants exhibited typical symptoms of eutypiosis including dwarf shoots, bushy phenotype with small chlorotic leaves, and marginal necrosis (Fig. 2). The area close to the zone of necrosis was cut into sections and 20 fragments per plant were incubated on culture medium. Positive re-isolation was considered to have occurred when fungal growth was seen 10 d after the beginning of incubation. Table 2 gives, for each plant, the number of fragments for which fungal growth was obtained. Fungal infection (positive *E. lata* re-isolation) was confirmed for the 12 plants which showed symptoms every year of the survey. Among the 15 plants that never exhibited symptoms, seven never showed any fungal growth, whereas eight were contaminated. Other fungi (i.e. *Botryosphaeria obtusa*, *Phaeomoniella chlamydospora*, *Phaeoacremonium aleophilum*, and *Trichoderma* sp.) were also visually identified after re-isolation. Four plants for which the number of ‘positive’ fragments was ≥50% and devoid of infection by other fungi were selected and called S'R+ (symptoms+ re-isolation+). Four plants among those that did not yield growth of *E. lata*, *P. chlamydospora*, *P. aleophilum*, and *Trichoderma* sp. were considered as healthy plants and selected. These S'R- plants allowed some re-isolation of *Botryosphaeria*; this was also the case for two of the plants that were selected as S'R+. Thus, because it is present in both samples it can be assumed that the genes that were differentially expressed between S'R+ and S'R- samples are not due to interaction with *Botryosphaeria*.

**Table 2.** Identification in the Chateau Cruzeaux vineyard of putative healthy plants (no symptoms) and putative infected grapevines (visible symptoms) based on surveys between 2002 and 2006

The disease scale used is described by Darrieutort and Lecomte (2007) (A) and the results of respective isolation tests from wood lesions are shown (B). Bold (S'R+), italics (S'R-), and bold italics (S'R-) identify plants that were selected for microarray analysis.

| Plant (A) | *Eutypa* dieback symptoms notation | (B) Recovery results |
|-----------|-----------------------------------|---------------------|
|           |                                   | *E. lata* (rate/20) | *Botryosphaeria* | *P. chlamydospora* | *P. aleophilum* | *Trichoderma* |
|           |                                   |                    |                   |                   |                   |               |
| R18C38    | E2                                | 20                 | –                  | –                  | –                  | –              |
| R16C19    | E1 S1 S2 E4 E4                    | 17                 | –                  | –                  | –                  | –              |
| R8C45     | BM BM BM BM BM BM BM E1 BM E1    | 12                 | +                  | –                  | –                  | –              |
| R10C39    | U E1 R S1 O E1 U R E1            | 10                 | +                  | –                  | –                  | –              |
| R0C39     | E4 E2 E4 O E4 E2                 | 8                  | +                  | –                  | –                  | –              |
| R5C65     | BM BM BM BM BM BM E1 E3 E1 E3    | 8                  | +                  | +                  | +                  | –              |
| R18C56    | BM BM BM BM U R E2 U R E2/4     | 3                  | –                  | –                  | +                  | –              |
| R6C23     | BM BM BM BM BM E3 E3 E3 E3      | 2                  | –                  | –                  | –                  | +              |
| R4C4      | E1 E1 O E1 E2                    | 2                  | +                  | –                  | –                  | –              |
| R13C66    | BM BM BM BM BM E1 E1/3 E1 E1/3  | 1                  | –                  | +                  | +                  | –              |
| R11C20    | BM BM BM BM BM E1 E1            | 1                  | +                  | +                  | +                  | –              |
| R10C34    | BM BM BM BM BM E1 E1            | 1                  | +                  | +                  | +                  | –              |
| R18C12    | 0 0 0 0 0 0 0                    | 0                  | +                  | –                  | –                  | –              |
| R18C23    | 0 0 0 0 0 0 0                    | 0                  | +                  | –                  | –                  | –              |
| R13C8     | 0 0 0 0 0 0 0                    | 0                  | +                  | –                  | –                  | –              |
| R17C18    | 0 0 0 0 0 0 0                    | 0                  | +                  | –                  | –                  | –              |
| R12C47    | 0 0 0 0 0 0 0                    | 0                  | +                  | +                  | +                  | –              |
| R16C49    | 0 0 0 0 0 0 0                    | 0                  | +                  | +                  | –                  | –              |
| R17C22    | 0 0 0 0 0 0 0                    | 0                  | –                  | –                  | –                  | +              |
| R11C48    | 0 0 0 0 0 0 1                    | 1                  | +                  | –                  | –                  | –              |
| R8C60     | 0 0 0 0 0 0 2                    | 2                  | –                  | –                  | –                  | –              |
| R19C8     | 0 0 0 0 0 0 5                    | 5                  | –                  | +                  | –                  | –              |
| R4C68     | 0 0 0 0 0 0 6                    | 6                  | +                  | –                  | –                  | –              |
| R10C12    | 0 0 0 0 0 0 9                    | 9                  | +                  | –                  | –                  | –              |
| R18C9     | 0 0 0 0 0 0 10                   | 10                 | –                  | –                  | –                  | –              |
| R17C4     | 0 0 0 0 0 0 12                   | 12                 | +                  | –                  | –                  | –              |
| R20C3     | 0 0 0 0 0 0 14                   | 14                 | +                  | –                  | –                  | –              |

E1, mild symptoms on one arm; E2, mild symptoms on the other arm; E1/3, mild symptoms on both arms; E3, severe symptoms on one arm; E4, severe symptoms on the other arm; E2/4, severe symptoms on both arms; S1, weakly susceptible on one arm; S2, weakly susceptible on the other arm; S3, strongly susceptible on one arm; S4, strongly susceptible on the other arm; U, single arm; BM, dead arm; 0, healthy; R, restored.

Downloaded from https://academic.oup.com/jxb/article-abstract/61/6/1719/462759 by guest on 30 July 2018
Formal identification of E. lata in infected plants

Formal identification of *E. lata* in the re-isolation samples collected from infected greenhouse and vineyard plants was successfully achieved by the protocol of *Lardner et al.* (2005). This protocol is based on DNA extraction from the re-isolated mycelium, followed by PCR with the Eut02F3 and Eut02R2 primers. It allowed characterization of *E. lata* in all infected samples (S* R* + and S* R* +) selected from greenhouse and vineyard plants (Fig. 3). A DNA fragment of the expected size (643 bp) was amplified from the mycelium growing from all the infected fragments, and a pure *E. lata* strain (BX1-10, NE85-1). This extensive characterization of plant material either prepared in the greenhouse or collected in the vineyard allowed identification of three series of plants: healthy plants with no symptoms and no re-isolation of *E. lata* (S* R* ), infected plants from which the fungus was successfully re-isolated but that did not show *Eutypa* dieback symptoms (S* R* +), and infected plants (with successful re-isolation of *E. lata*) exhibiting eutypiosis symptoms (S* R* +). RNA was extracted from leaves of S* R* , S* R* +, and S* R* + plants, and used for hybridization with the 15 K Qiagen/operon microarray.

**Microarray analysis**

Analysis of the microarrays was conducted from infected plants with symptoms (S* R* +), infected plants without symptoms (S* R* +), and healthy plants (S* R* ).

The microarray data were first used to identify genes that were differentially expressed between infected plants with symptoms (S* R* +) and healthy (S* R* ) plants. In order to increase the stringency of the differentially expressed genes and to identify the most interesting genes that characterize grapevine response to *E. lata*, the microarray data produced from greenhouse and vineyard (S* R* +) and (S* R* ) material described herein were combined with microarray data that we obtained previously with *in vitro* plantlets experimentally infected by *E. lata* (accession number E-MEXP-2102 in Array Express; *Rotter et al.*, 2009).

**Fig. 2.** Cabernet-Sauvignon grapevine naturally infected in the vineyard. (A) Leaf symptoms. (B) Typical sectorial necrosis from which *E. lata* mycelium may be re-isolated (C).

**Fig. 3.** Indirect PCR identification of the presence of *E. lata* in vineyard and greenhouse plants. The tested samples are mycelia growing from S* R* + and S* R* + greenhouse plants infected with the BX1-10 *E. lata* strain and from vineyard S* R* + and S* R* + plants. The PCR was also run either with DNA from BX1-10 and NE85-1 pure mycelia (positive control, C+) or with water as matrix (negative control, C−).

**Fig. 4.** Microarray experimental design. The microarray data produced with greenhouse and vineyard material described herein were combined with data that we obtained previously in *in vitro* conditions (*Rotter et al.*, 2009). Three kind of plants were characterized: infected with symptoms (S* R* +), infected without symptoms (S* R* ), and healthy (S* R* ). Three comparisons were performed (S* R* /S* R* ), (S* R* /S* R* ), and (S* R* /S* R* +). For each comparison the number of the biological replicate (BR) and the number of technical replicates corresponding to the dye swap between cyanine 5 and cyanine 3 (TR) is specified.
The microarray data were also used to identify genes that may be involved in the lack of symptoms, and thus may play some role in the tolerance to *E. lata*. For this, comparisons were made between $S^R+/S^R+$ plants, and between $S^R+/S^R-$ plants produced in both greenhouse and vineyard conditions. An overview of the microarray experimental design is presented in Fig. 4.

**Identification of genes differentially expressed between infected plants with eutypiosis symptoms and healthy plants ($S^R+/S^R-$)**

Genes differentially expressed between $S^R+$ and $S^R-$ plants were identified in three experimental conditions *in vitro*, in the greenhouse, and in the vineyard. Only a few genes were differentially expressed if thresholds of 2 for up-regulation and 0.5 for down-regulation were set, with a $P$-value $<0.05$. The numbers of up- and down-regulated genes were 25, 70, and 131, and 1, 35, and 45, respectively, in *in vitro*, greenhouse, and vineyard conditions. These low figures may be due to the fact that the major impact of the vascular fungus *Eutypa* on xylem tissue is diluted when whole leaf samples are analysed. However, it was technically impossible to extract RNA from the xylem of lignified

---

**Fig. 5.** Venn diagram showing the distribution of genes differentially expressed ($P$-value $\leq 0.05$ and threshold $\geq 1.5$) between infected plants with symptoms ($S^R+$) and healthy plants ($S^R-$) grown *in vitro*, in the greenhouse, and in the vineyard. The numbers of up- and down-regulated genes in infected plants with symptoms ($S^R+$) compared with healthy plants ($S^R-$) are indicated in bold and italics, respectively. The number of differentially expressed genes that are found in common between several growth conditions is underlined at the intersection of the corresponding circles. Total numbers refer to up- and down-regulated for a given growth condition.

**Fig. 6.** Distribution into functional categories of genes differentially expressed between $S^R+$ and $S^R-$ plants ($P$-value 0.05 and threshold 1.5) in at least two growth conditions. Only the genes showing a good homology with known genes were considered. The number of genes of each category is reported on the abscissa. The genes repressed in infected plants with symptoms are shown by a cross-hatched bar when they are common to greenhouse and vineyard conditions or by a grey bar when they are common to *in vitro*, greenhouse, and vineyard conditions. The genes which are up-regulated in these plants are represented by a black bar when they are common to *in vitro*, greenhouse, and vineyard conditions, by a bar with thick diagonal lines when they are common to *in vitro* and greenhouse conditions, by a bar with thin diagonal lines when they are common between greenhouse and vineyard conditions, and by a white bar when they are common to *in vitro* and vineyard conditions.
Table 3. Functional classification of the genes differentially expressed (ratio $\geq 1.5$ or $\leq 0.66$ and $P$-value $< 0.05$) between $S^+R^+$ and $S^-R^-$ plants, for at least two conditions: in vitro (I), in the greenhouse (G), or in the vineyard (V), and showing a good homology with known genes.

The grapevine genome identifier (G8X ID), the DFCI grape gene index version 6 identifier (VvGI6 ID), and the protein ID associated with these sequences are given in Supplementary Table S2 at JXB online.

| Probe ID | Annotation | Profile | Condition | In vitro (I) | Greenhouse (G) | Vineyard (V) |
|----------|------------|---------|-----------|--------------|---------------|--------------|
|          |            | Regulation | Ratio | $P$-value | Ratio | $P$-value | Ratio | $P$-value |
| **Extracellular metabolism** |            |           |          |            |              |              |        |          |
| Vv_10002068 | Homologue to $\beta$-1,3-glucanase complete | Up | I+G+V | 1.864 | 3.95E-07 | 1.5568 | 0.00256 | 2.9927 | 2.6E-05 |
| Vv_1000389 | Similar to $\beta$-1,3-glucanase complete | Up | I+G+V | 2.005 | 2.27E-07 | 1.5752 | 0.003244 | 1.7724 | 2.4E-07 |
| Vv_10010418 | Similar to $\beta$-1,3-glucanase complete | Up | I+G+V | 2.057 | 5.79E-08 | 4.0505 | 0.000441 | 6.9477 | 1.6E-05 |
| Vv_10004763 | Weakly similar to germin-like protein partial (88%) | Down | G+V | 1.122 | 0.097133 | 0.6458 | 0.003705 | 0.5948 | 7.7E-06 |
| **Amino acid metabolism** |            |           |          |            |              |              |        |          |
| Vv_10008453 | Homologue to putative serine hydrolase complete | Up | I+G+V | 1.514 | 3.08E-05 | 2.9137 | 0.003491 | 1.8146 | 1.9E-06 |
| Vv_10005036 | Similar to alanine–glyoxylate aminotransferase complete | Up | G+V | 0.905 | 0.009552 | 1.6602 | 0.003996 | 1.6719 | 9.5E-06 |
| Vv_10001606 | Similar to asparagine synthetase complete | Up | G+V | 0.962 | 0.002379 | 1.7886 | 0.001989 | 1.9321 | 9E-07 |
| Vv_10004099 | Similar to asparagine synthase-related protein complete | Up | G+V | 1.051 | 0.829175 | 2.848 | 0.007312 | 3.3818 | 4.2E-08 |
| **Phenylpropanoid metabolism** |            |           |          |            |              |              |        |          |
| Vv_10004786 | Similar to acyl:CoA ligase complete | Up | G+V | 0.978 | 0.009565 | 0.6392 | 0.002503 | 0.6429 | 1.9E-06 |
| Vv_100011235 | Weakly similar to tetrahydroxychalcone 2'-glucosyltransferase complete | Up | G+V | 0.962 | 0.002379 | 1.7886 | 0.001989 | 1.9321 | 9E-07 |
| Vv_10002511 | Similar to flavanone 3-hydroxylase-like protein complete | Up | G+V | 1.411 | 0.005707 | 1.6943 | 0.006336 | 3.1096 | 7E-07 |
| **Carbon metabolism** |            |           |          |            |              |              |        |          |
| Vv_10004223 | Weakly similar to ketose-bisphosphate aldolase partial (96%) | Up | G+V | 0.876 | 0.003581 | 1.6469 | 0.001954 | 2.7734 | 2.4E-07 |
| Vv_10003661 | Similar to sucrose synthase complete | Up | G+V | 1.025 | 0.510806 | 1.7368 | 0.001049 | 1.8316 | 1E-06 |
| Vv_1000306 | Weakly similar to $\beta$-amylase complete | Up | G+V | 0.869 | 0.009564 | 2.1877 | 0.024777 | 1.8991 | 4.6E-07 |
| Vv_10003065 | Similar to putative fructokinase-5 complete | Down | G+V | 0.978 | 0.005656 | 0.6392 | 0.002503 | 0.6429 | 1.9E-06 |
| **Lipid metabolism** |            |           |          |            |              |              |        |          |
| Vv_10013248 | Weakly similar to GDSL esterase/lipase partial (93%) | Down | G+V | 0.884 | 0.009755 | 0.558 | 0.000822 | 0.5972 | 1.7E-07 |
| Vv_10000536 | Similar to GDSL esterase/lipase partial (94%) | Down | G+V | 0.851 | 0.001213 | 0.4415 | 0.005116 | 0.6303 | 1.8E-06 |
| Vv_10008537 | Weakly similar to GDSL esterase/lipase partial (93%) | Down | G+V | 0.97 | 0.659092 | 0.6076 | 0.007392 | 0.619 | 4.8E-07 |
| **Metabolism** |            |           |          |            |              |              |        |          |
| Vv_10007334 | Weakly similar to cytochrome P450 complete | Up | G+V | 1.021 | 0.55331 | 1.5366 | 0.00442 | 2.4597 | 1.2E-06 |
| Vv_10004967 | Weakly similar to cytochrome P450 partial (95%) | Up | G+V | 0.984 | 0.376553 | 1.8565 | 0.004331 | 1.7544 | 1E-06 |
| **Biogenesis of cellular compounds: cell wall** |            |           |          |            |              |              |        |          |
| Vv_10009806 | Similar to fasciclin-like AGP 11 partial (62%) | Down | G+V | 1.275 | 0.000147 | 0.5284 | 0.002599 | 0.6409 | 3.2E-05 |
| Vv_1001696 | Weakly similar to fasciclin-like AGP 11 partial (63%) | Down | G+V | 1.256 | 0.000394 | 0.5096 | 0.0032 | 0.6098 | 3.1E-06 |
| Vv_10010533 | Homologue to expansin complete | Down | G+V | 1.076 | 0.211971 | 0.6241 | 0.002505 | 0.5829 | 1.9E-07 |
| Vv_1004211 | Similar to xyloglucan endotransglycosylase partial (96%) | Down | G+V | 0.872 | 0.001466 | 0.4261 | 0.000497 | 0.4719 | 9.3E-08 |
| Vv_10011060 | Weakly similar to HyPP2 partial (94%) | Up | G+V | – | – | 3.9942 | 1.62E-06 | 3.3677 | 6.3E-08 |
| Vv_10011061 | Weakly similar to HyPP2 partial (94%) | Up | G+V | – | – | 4.6746 | 0.000207 | 2.7843 | 3.1E-07 |
| Vv_1001712 | Similar to XET complete | Up | G+V | 1.017 | 0.803499 | 1.669 | 0.005678 | 1.8456 | 0.0233 |
| **Defence response** |            |           |          |            |              |              |        |          |
| Vv_10005843 | Weakly similar to pectin methylesterase inhibitor-like protein complete | Down | I+V | 0.554 | 1.67E-06 | 1.4363 | 0.006723 | 0.3711 | 4.3E-08 |
| Vv_10003874 | Homologue to pathogenesis-related protein 10 complete | Up | I+G+V | 1.856 | 0.00029 | 3.8531 | 0.000153 | 8.3957 | 3.6E-07 |
| Probe ID     | Annotation                                      | Profile                                      | In vitro (I) | Greenhouse (G) | Vineyard (V) |
|-------------|-------------------------------------------------|----------------------------------------------|-------------|---------------|-------------|
| Vv_10010887 | Homologue to pathogenesis-related protein 10.3 partial (58%) | Up I+G+V                                    | 1.801       | 0.005897      | 3.0526      |
| Vv_10011243 | Homologue to putative pathogenesis-related protein 1 partial (88%) | Up I+G+V                                    | 2.372       | 2.3E-06       | 1.7636      |
| Vv_10004985 | Similar to putative pathogenesis-related protein 1 partial (98%) | Up I+G+V                                    | 2.503       | 1.42E-05      | 1.6072      |
| Vv_10004683 | Similar to NtPPrp27 partial (89%)               | Up G+V                                      | 1.381       | 0.002058      | 2.031       |
| Vv_10009597 | Weakly similar to hairpin-inducing protein complete | Up I+G+V                                    | 2.367       | 1.2E-07       | 5.3487      |
| Vv_10000872 | Similar to rheumatin-like protein partial (93%)  | Up I+G                                      | 1.585       | 8.8E-08       | 2.8029      |
| Vv_1000136  | Homologue to class IV chitinase partial (92%)   | Up G+V                                      | 2.677       | 2.7E-06       | 7.4383      |
| Vv_10002903 | Similar to class IV chitinase partial (94%)     | Up G+V                                      | 2.18        | 4.5E-08       | 2.3405      |
| Vv_10004018 | Similar to class IV chitinase complete           | Up I+G                                      | 1.514       | 4.81E-05      | 1.6071      |
| Vv_10000957 | Weakly similar to glutathione S-transferase GST 18 complete | Up G+V                                    | 1.144       | 0.001318      | 1.5802      |
| Vv_10000136 | Homologue to class IV chitinase partial (92%)   | Up G+V                                      | 2.367       | 1.29E-07      | 2.8228      |
| Vv_10000872 | Similar to rheumatin-like protein partial (93%)  | Up I+G                                      | 1.585       | 8.8E-08       | 2.8029      |
| Vv_10000872 | Similar to rheumatin-like protein partial (93%)  | Up I+G                                      | 1.585       | 8.8E-08       | 2.8029      |
| Vv_10000957 | Weakly similar to glutathione S-transferase GST 18 complete | Up G+V                                    | 1.144       | 0.001318      | 1.5802      |
| Vv_10000136 | Homologue to class IV chitinase partial (92%)   | Up G+V                                      | 2.367       | 1.29E-07      | 2.8228      |
| Vv_10000872 | Similar to rheumatin-like protein partial (93%)  | Up I+G                                      | 1.585       | 8.8E-08       | 2.8029      |
| Vv_10000957 | Weakly similar to glutathione S-transferase GST 18 complete | Up G+V                                    | 1.144       | 0.001318      | 1.5802      |
| Vv_10000136 | Homologue to class IV chitinase partial (92%)   | Up G+V                                      | 2.367       | 1.29E-07      | 2.8228      |
| Vv_10000872 | Similar to rheumatin-like protein partial (93%)  | Up I+G                                      | 1.585       | 8.8E-08       | 2.8029      |
| Vv_10000957 | Weakly similar to glutathione S-transferase GST 18 complete | Up G+V                                    | 1.144       | 0.001318      | 1.5802      |
concerns only three categories (Fig. 6). Five categories are represented in overexpressed genes, whereas underexpression allows the establishment of an expected expression profile between the different kinds of plants: S+R+, S+R, and S+R−. For greenhouse plants, 26 genes were overexpressed and six genes were down-regulated in S+R− plants compared with S+R+ and S+R− plants; for vineyard plants, 49 genes were overexpressed and 10 genes were repressed in S+R+ plants compared with S+R+ and S+R− plants.

The genes that may be involved in the absence of symptom development in greenhouse or vineyard conditions, which exhibited good homology with genes of known function, are listed in Table 4, and arranged by functional categories (Fig. 7). Among the genes that may be assigned to functional categories (34 up-regulated genes and five down-regulated genes in total), the most abundant belong to the category of energy metabolism, and more precisely to the light phase of photosynthesis. All those genes were up-regulated (Fig. 7). Four of them encode subunits of NADH-plastoquinone oxidoreductase, four encode other membrane proteins of the photosynthetic apparatus (oxygen-evolving enhancer protein 2, cytochrome b6, PSI chlorophyll a/b-binding protein, and PSII CP47 chlorophyll apoprotein),...
Table 4. Functional classification of the genes more specifically associated with absence of symptoms

These genes are differentially expressed (ratio \(>1.4\) or \(<0.71\) and \(P\)-value < 0.05) exclusively in both comparisons of the S‘R+ /S‘R+ and S‘R+/S‘R–, they are identified in greenhouse (G) and/or in vineyard (V) conditions, and they show a good homology with known genes. The grapevine genome identifier (G8X ID), the DFCI grape gene index version 6 identifier (VvGI6 ID), and the protein ID associated with these sequences are given in Supplementary Table S3 at JXB online.

| Probe ID       | Annotation                                      | Profile | S‘R+/S‘R+ | S‘R+/S‘R– | S‘R+/S‘R– |
|----------------|-------------------------------------------------|---------|-----------|-----------|-----------|
| Lipid metabolism |                                                |         |           |           |           |
| Vv_10000444    | Weakly similar to GDSL esterase/lipase partial (91%) | Down    | G         | 0.703     | 0.01226   | 0.659     | 0.00015   | 0.824     | 0.04918 |
| Phenylpropanoid metabolism |                                     |         |           |           |           |
| Vv_10000352    | Similar to anthocyanidin synthase complete       | Up      | V         | 1.534     | 0.01083   | 1.517     | 0.00645   | 0.898     | 0.01678 |
| Vv_10003777    | Similar to anthocyanidin synthase complete       | Up      | V         | 1.790     | 0.00481   | 1.560     | 0.00927   | 0.749     | 0.00602 |
| Vv_10010748    | Homologue to chalcone synthase complete          | Up      | V         | 1.603     | 0.00759   | 1.462     | 0.01094   | 0.875     | 0.00970 |
| Vv_10004167    | Homologue to chalcone synthase complete          | Up      | V         | 1.629     | 0.00694   | 1.407     | 0.01531   | 0.878     | 0.04273 |
| Phenylpropanoid metabolism |                                     |         |           |           |           |
| Vv_10007239    | Similar to trehalose-phosphate phosphatase complete | Up      | V         | 1.520     | 0.01082   | 1.448     | 0.00974   | 0.892     | 0.00615 |
| Vv_10010928    | Fructose-bisphosphate aldolase complete          | Up      | V         | 2.220     | 0.00162   | 1.474     | 0.01235   | 0.752     | 0.00294 |
| Vv_1000154     | Fructose-bisphosphate aldolase complete          | Up      | V         | 2.149     | 0.02130   | 1.450     | 0.01555   | 0.712     | 0.00163 |
| Vv_10000002    | Similar to galactinol synthase partial (96%)     | Up      | G         | 1.468     | 0.02546   | 1.736     | 0.00022   | 1.563     | 0.01526 |
| Carbon metabolism |                                                |         |           |           |           |
| Vv_10000953    | Glutamine synthetase partial (97%)               | Up      | V         | 1.752     | 0.00512   | 1.459     | 0.01092   | 0.8438    | 0.00858 |
| Vv_10000162    | Similar to NAD(P)H-quinone oxidoreductase subunit 6 partial (89%) | Up      | V         | 1.761     | 0.00924   | 1.832     | 0.00213   | 0.857     | 0.07298 |
| Vv_10010684    | Homologue to NAD(P)H-quinone oxidoreductase subunit H, chloroplastic partial (87%) | Up      | V         | 1.563     | 0.00930   | 1.661     | 0.00222   | 0.786     | 0.04834 |
| Vv_10010940    | Homologue to NAD(P)H-quinone oxidoreductase subunit 1 chloroplastic complete | Up      | G+V      | 1.596     | 0.00531   | 1.402     | 0.00308   | 1.048     | 0.54933 |
| Vv_10000222    | Similar to oxygen-evolving enhancer protein 2 complete | Up      | V         | 1.627     | 0.01653   | 1.443     | 0.02712   | 0.724     | 0.00581 |
| Vv_1000172     | Similar to type III chlorophyll a/b-binding protein partial (95%) | Up      | V         | 1.653     | 0.00624   | 1.477     | 0.02434   | 0.776     | 0.00033 |
| Vv_10011239    | Photosystem II CP47 chlorophyll apoprotein complete | Up      | V         | 1.483     | 0.02012   | 1.898     | 0.00161   | 0.947     | 0.04738 |
| Vv_10008623    | Weakly similar to thylakoid luminal 16.5 kDa protein partial (68%) | Up      | V         | 1.800     | 0.01531   | 1.469     | 0.04659   | 0.740     | 0.00061 |
| Vv_1004046     | Weakly similar to thioredoxin M complete          | Up      | G         | 1.471     | 0.00372   | 1.456     | 0.00020   | 0.939     | 0.64997 |
| Vv_10012092    | Weakly similar to RbcX protein partial (64%)      | Up      | G+V      | 1.431     | 0.00592   | 1.431     | 0.00020   | 0.971     | 0.68974 |
| Vv_10008508    | Homologue to phosphoribulokinase complete         | Up      | V         | 1.683     | 0.00562   | 1.422     | 0.01666   | 0.749     | 0.00087 |
| Vv_10004505    | ABC-ATPase, partial (89%)                        | Down    | G         | 1.543     | 0.01076   | 1.408     | 0.01437   | 0.943     | 0.07910 |
| Protein synthesis |                                                |         |           |           |           |
| Vv_10011319    | Weakly similar to 50S ribosomal protein L16, chloroplastic (fragment) complete | Up      | V         | 2.897     | 0.01015   | 1.625     | 0.03328   | -         | -         |
| Vv_10001754    | Similar to 30S ribosomal protein S1, chloroplastic partial (69%) | Up      | V         | 1.617     | 0.00790   | 1.401     | 0.01520   | 0.838     | 0.00479 |
| Protein activity |                                                |         |           |           |           |
| Vv_1004810     | Weakly similar to FKBP-type peptidyl-prolyl cis-trans isomerase partial (77%) | Up      | V         | 1.405     | 0.02036   | 1.438     | 0.01256   | 0.900     | 0.03535 |
| Vv_10013654    | Similar to putative FKBP type peptidyl-prolyl cis-trans isomerase complete | Up      | V         | 1.677     | 0.00625   | 1.466     | 0.00862   | 0.780     | 0.00004 |
| Vv_1002247     | Weakly similar to cysteine protease partial (90%) | Up      | V         | 2.096     | 0.00273   | 1.460     | 0.01143   | 0.766     | 0.00314 |
| Transport |                                                |         |           |           |           |
| Vv_10011055    | Weakly similar to non-specific lipid-transfer protein type 2 complete | Up      | G         | 2.362     | 0.00044   | 1.564     | 0.03055   | 0.722     | 0.15242 |
and three encode soluble proteins (RBCX, phosphoribulokinase, and thioredoxin) (Table 4). Besides energy metabolism (photosynthesis), other functional categories seemed to be linked to lack of symptom development. They included phenylpropanoid metabolism, carbon metabolism, protein synthesis or regulation, defence reactions, and cell wall metabolism.

Validation of candidate genes by RT-PCR

Of the 26 genes that were up-regulated in S +R+ plants, eight were selected to study their expression by RT-PCR. These genes code for osmotin (Vv-10010885: GSVIVG00001106001), PR10 protein (Vv-10003874: GSVIVG00033089001), chitinase (Vv-10000136: GSVIVG00034644001), tumour-related protein (Vv-10001691: GSVIVG00007741001), disease resistance response protein (Vv-10010268: GSVIVG00024743001), harpin-induced protein (Vv-10009597: GSVIVG00021517001), legumin (TC72587), and a small proline-rich protein (GSVIVG00034255001). The elongation factor EF1 was used as a constitutive control. The transcripts of the eight selected genes were more abundant in infected symptomatic plants (S +R+) than in healthy plants (S–R–). To check the specificity of the response of these genes, their expression was also studied in plants infected by either downy mildew, powdery mildew, or black rot (Fig. 8 B). All the genes were also up-regulated upon infection by these three fungi, indicating that they are general markers of fungal infection which are not specific for E. lata.

Discussion

Very few studies have been devoted to the interaction between a plant and a vascular pathogenic fungus (Dowd et al., 2004; Robb et al., 2007). To our knowledge, this paper provides the first transcriptomic analysis of the interaction of grapevine with the causal agent of Eutypa dieback, a major vascular disease.

Characterization of plant material

In the vineyard, Eutypa symptoms appear several years after infection (Duthie et al., 1991; Tey-Rulh et al., 1991), and for a given plant the symptoms are variable from one year to the next, even after the symptoms have appeared for the first time. This makes this disease very hard to study. For these reasons, transcriptomic analyses were carried out with plants that were carefully characterized after symptom notation and fungus isolation, in order to distinguish infected plants with typical Eutypa symptoms (S’R’), infected plants without visible symptoms (S’R”), and healthy plants (S R”). The symptoms observed 1 year after inoculation of greenhouse cuttings, which included stunting of new shoots, with small, cupped, chlorotic, and tattered leaves, were also observed in several other greenhouse studies: 14 months after infection of rooted grapevine cutting inoculated with E. lata ascospores (Pezoldt et al., 1981), 4–8 weeks after inoculation of unrooted cuttings

---

Table 4. Continued

| Probe ID   | Annotation                                                                 | Condition     | Ratio | P-value | Ratio | P-value | Ratio | P-value |
|------------|------------------------------------------------------------------------------|---------------|-------|---------|-------|---------|-------|---------|
| W_10000356 | Similar to SNF-related protein kinase regulatory subunit 1 complete         | Up            | 1.49  | 0.0038  | 1.22  | 0.0006  | 1.22  | 0.085   |
| W_10000537 | Homologue to alcohol dehydrogenase complete                                  | Up            | 1.45  | 0.0083  | 1.74  | 0.0002  | 1.29  | 0.036   |
| W_10000538 | Similar to class IV chitinase partial                                         | Up            | 1.39  | 0.0013  | 0.89  | 0.0006  | 1.24  | 0.003   |
| W_10000539 | Similar to peroxiredoxin partial (97%)                                       | Up            | 1.41  | 0.0003  | 0.89  | 0.0006  | 1.24  | 0.003   |
| W_10000540 | Similar to thioredoxin peroxidase partial (97%)                              | Up            | 1.45  | 0.0083  | 1.74  | 0.0002  | 1.29  | 0.036   |
| W_10000541 | Similar to class IV chitinase partial                                         | Up            | 1.39  | 0.0013  | 0.89  | 0.0006  | 1.24  | 0.003   |
| W_10000542 | Similar to peroxiredoxin partial (97%)                                       | Up            | 1.41  | 0.0003  | 0.89  | 0.0006  | 1.24  | 0.003   |
| W_10000543 | Similar to thioredoxin peroxidase partial (97%)                              | Up            | 1.45  | 0.0083  | 1.74  | 0.0002  | 1.29  | 0.036   |
| W_10000544 | Similar to class IV chitinase partial                                         | Up            | 1.39  | 0.0013  | 0.89  | 0.0006  | 1.24  | 0.003   |
| W_10000545 | Similar to peroxiredoxin partial (97%)                                       | Up            | 1.41  | 0.0003  | 0.89  | 0.0006  | 1.24  | 0.003   |
| W_10000546 | Similar to thioredoxin peroxidase partial (97%)                              | Up            | 1.45  | 0.0083  | 1.74  | 0.0002  | 1.29  | 0.036   |
| W_10000547 | Similar to class IV chitinase partial                                         | Up            | 1.39  | 0.0013  | 0.89  | 0.0006  | 1.24  | 0.003   |
| W_10000548 | Similar to peroxiredoxin partial (97%)                                       | Up            | 1.41  | 0.0003  | 0.89  | 0.0006  | 1.24  | 0.003   |
maintained in moist rockwool with an *E. lata* mycelium plug (Peros *et al.*, 1994, 1999), or 8 months after infection of rooted cuttings with an *E. lata* mycelium plug (Sosnowski *et al.*, 2007). Isolation of the fungus present in woody tissues and PCR identification of *E. lata* were also carried out to characterize the plant material. Numerous DNA-based markers are available to identify *E. lata* (Lecomte *et al.*, 2000; Rolshausen *et al.*, 2004; Lardner *et al.*, 2005; Catal *et al.*, 2007). The SCAR primer pair Eut02 F3/Eut02 R2 (Lardner *et al.*, 2005) was used in the present study. The development of *E. lata* PCR primers is very interesting because it allows a formal *E. lata* diagnosis test. However, this is a destructive assay requiring the use of perennial grapevine wood tissues. The different tests made allowed checks to be made to determine whether the uninoculated control or the grapevines that seemed to be healthy were indeed axenic, and to separate the experimentally inoculated samples that became infected from those that did not.

**Microarray analysis**

Eutypiosis is also hard to study because each possible experimental model (*in vitro*, greenhouse, or vineyard) has specific advantages and disadvantages. Vineyard plants infected with *E. lata* obviously represent the closest material to natural conditions, but the infection process and the environment are not controlled. In this study, the status of naturally infected vineyard plants was monitored for several years. Greenhouse and *in vitro* plants can be experimentally infected. In this study, greenhouse and *in vitro* plants were inoculated with a characterized *E. lata* strain under a controlled environment. *Eutypa* symptoms appeared after 1 year for greenhouse plants and after only 7 weeks for *in vitro* plants. However, greenhouse cuttings are a simplified model and *in vitro* plants do not differentiate much woody tissue, which makes this material less close to natural conditions. Furthermore, although it is thought that grapevine infection by *E. lata* occurs through wounds in natural conditions (Carter, 1960, 1965; Moller *et al.*, 1978), infection via a cut stem or a stem hole may not completely reflect the natural sequence of events. Notwithstanding this, great care was taken to check the physiological status of each series of plants.

It is because each experimental condition presents specific advantages and disadvantages that transcriptomic analyses were carried out on the three experimental conditions (*in vitro*, greenhouse, and vineyard) and that the data were combined in order to determine only the most significant genes.

**Fig. 7.** Distribution into functional categories of differentially expressed genes which are associated with lack of symptoms. These genes are differentially expressed (threshold ≥1.4, *P*-value ≤0.05) in greenhouse and vineyard plants, and common to S'R'/S'R' and S'R'/S'R' comparisons between S'R' and S'R' plants (*P*-value 0.05 and threshold 1.5) in at least two growth conditions. Only the genes showing a good homology with known genes were considered. The number of genes of each category is reported on the abscissa. The genes repressed in infected plants without symptoms are shown by a cross-hatched bar for vineyard conditions or by a white bar in greenhouse conditions. The genes which are up-regulated in these plants are represented by a black bar when they are common to greenhouse and vineyard conditions, by a bar with thick diagonal lines for greenhouse conditions, and by a bar with thin diagonal lines for vineyard conditions.
Due to the impossibility of RNA isolation from lignified vascular tissues, it was decided to analyse leaf samples, because RNA can be easily extracted from leaves and because leaves exhibit dramatic symptoms in the case of infection. The ratios observed for differential expression were rather low and led to low thresholds being used in most cases. Possible reasons for this are the dilution of infected zones of leaves with healthy leaf parts, the choice of leaf samples while the first invaded tissue is the xylem, and the long times chosen for sampling.

Comparison between infected plants with symptoms and healthy plants

The number of up- and down-regulated genes in infected plants with symptoms compared with healthy plants increased from *in vitro* to greenhouse and vineyard conditions. Part of this observation might be explained by the kinetics of infection. Indeed, the contact between the grapevine and *E. lata* lasts 7 weeks *in vitro*, 1 year in the greenhouse, and 5 years in the vineyard. The material produced *in vitro* and in the greenhouse corresponds to earlier steps of infection than that in the vineyard. Microarray studies conducted on other plant pathogen systems also revealed that the number of genes differentially expressed increased during infection kinetics (Moy *et al.*, 2004; Zhao *et al.*, 2007; Fung *et al.*, 2008). Another explanation may be that the environment is less controlled and stable between *in vitro*, greenhouse, and vineyard conditions.

The number of up-regulated genes was much higher than the number of repressed genes. The same trend was observed after infection of tomato plants with *Verticillium dahliae* (Robb *et al.*, 2007) or after treatment of tomato leaves with fusicoccin, a toxin secreted by *Fusicoccum amygdali* (Frick *et al.*, 2002). The response of the plant to fungal infection is therefore oriented more towards the stimulation of specific metabolic pathways than to the cessation of given processes.

According to the literature or to the pathoplant database (http://www.pathoplant.de/microarray.php), 44% (30/66 up-regulated, 4/11 down-regulated) of the genes differentially expressed in infected plants showing symptoms in at least two experimental conditions (Table 3) are already known to be involved in plant–fungus interaction. This result confirms the validity of the present approach. The gene *BIG8.1* (Vv_10008453: GSVIVG00032646001) encoding a serine hydrolase (AAN77692) was cloned after differential screening of transcripts expressed in grape leaves infected by *B. cinerea*, and its up-regulation by infection was confirmed by RT-PCR (Bezier *et al.*, 2002). The gene *CYP82H1* (Vv_10007334: GSVIVG00036466001) encoding the cytochrome P450 protein (Q6QNI1) is expressed more after elicitation by fungal extracts, and is thus probably involved in defence response (Larbart, 2006). The genes GSVIVG00002773001 (Vv_10001736) and...

---

**Fig. 8.** RT-PCR expression analysis for candidate genes selected from the microarray analysis. The genes selected are all up-regulated in S’R*+ plants compared with S’R* plants, for all the three types of conditions tested. (A) Response to *E. lata*. The expression was studied with the same plants as those used for microarray analysis. (B) Response to other pathogens: *E. necator*, *P. viticola*, and *B. cinerea*. OS, MS, and BS, control uninoculated plants; OI, plants collected 12 d or 14 d after inoculation with *E. necator*; MI, plants collected 12, 14, or 16 d after inoculation by *P. viticola*; BI, plants collected 1, 2, or 3 h after inoculation by *B. cinerea*.
GSVIVG00027001001 (Vv_10001880) are associated with the transcription factors CaWRKY-b (AY743433) and VaWRKY 30 (AY509152). Both these transcription factors are over-expressed in *V. vinifera* leaves of a susceptible cultivar infected with *E. necator* compared with healthy grapevine leaves (Fung et al., 2007). Both GSVIVG0001107001 (Vv_10003617) and GSVIVG0001106601 (Vv_10010885) are highly homologous to a *V. vinifera* gene encoding an osmotin (P93621). This protein has a strong antifungal activity and stops the mycelial growth of *Phomopsis viticola* and *B. cinerea*. It inhibits spore germination and germ tube growth of *E. necator*, *P. viticola*, and *B. cinerea*. Both gene expression and protein production are induced in grapevine leaves and berries infected by *E. necator* or *P. viticola* (Monteiro et al., 2003). Following leaf infection by *E. necator*, this gene is strongly induced in the resistant grapevine cultivar Regent compared with the susceptible variety Chardonnay (Leocir Welter, personal communication). *VvPR10*-1 (Vv_10003874: GSVIVG00033089001) encodes a pathogenesis-related protein PR10 (Q9FS42) which is induced in the leaves of the grapevines cultivar Riesling and Glory infected with the fungus *P. viticola* or *P. cubensis* (Kortekamp, 2006). This gene is also overexpressed in the Régent cultivar during the incompatible interaction between grapevine and *E. necator* (Leocir Welter, personal communication). *VvCHIT4c* (Vv_10002903: GSVIVG00034632001) encoding a class IV chitinase (Q7XB39), *VvPIN* (Vv_10008543: GSVIVG00029889001) encoding a protease inhibitor (Q6YEY6), and the gene (Vv_10002903: GSVIVG00033125001) coding for a *β*-1,3-glucanase (Q9M563) are all induced in elicited grapevine leaves or cells, and this treatment promotes resistance to the fungi *B. cinerea*, *E. necator*, and *P. viticola* (Aziz et al., 2003, 2004; Belhadj et al., 2006). GSVIVG00025341001 (Vv_100002068) and GSVIVG00025340001 (Vv_10000389) are associated with a second *β*-1,3-glucanase (Q9M3U4) whose transcripts are accumulated in the susceptible variety ‘Gloire de Montpellier’ after infection with *P. viticola* (Kortekamp, 2006).

All these responses tend to strengthen the plant cell wall (anionic peroxidase, proline-rich and hydroxyproline-rich proteins), to maintain the osmotic balance (osmotin, PR10, chitinase, tumour-related protein, and legumin). The RT-PCR profiles obtained for some genes are in agreement with literature data. Thus, the GSVIVG0001106601 (Vv_10010885) associated with an osmotin gene is up-regulated by infection with *E. necator* and *P. viticola* as observed by Monteiro et al. (2003). *VvPR10*-1 (Vv_10003874: GSVIVG00033089001) is up-regulated by *P. viticola*, as observed by Kortekamp (2006), and by *E. necator* (Leocir Welter, personal communication). To our knowledge, the other genes tested have not been shown to be involved in the response to infection by *E. necator*, *P. viticola*, or *B. cinerea* before this work.

**Energy metabolism and photosynthesis function seem to be particularly linked to lack of eutypiosis symptoms**

All the transcripts that were differentially expressed in the greenhouse or vineyard for both of the comparisons (S/R+ and S/R S R R) were considered together in order to identify genes that may prevent the development of the fungus and/or the symptoms (Fig. 7, Table 4).

Among the 91 genes whose differential expression correlated with lack of symptoms, 40 could be categorized into functional categories (Table 4). Out of these 40 genes, 10 were involved in light capture and electron transport in the chloroplast. This result may be related to the mode of action of *E. lata*'s toxins at the cellular level. Indeed, eutypine and the toxic polypeptide fraction secreted by *E. lata* behaved like protonophores that affect both structure and function of mitochondrial (Deswarte et al., 1996), plastidial (Deswarte et al., 1994), and plasma membranes (Amborabé et al., 2001; Octave et al., 2006a). Ultrastructural observations depicting a chloroplast swelling with a thylakoid dilatation (Deswarte et al., 1994) showed that eutypine also inhibits photosynthesis and interacts with the thylakoid membranes. Eutypine also uncouples mitochondrial oxidative phosphorylation in grapevine and potato cells (Deswarte et al., 1996). The toxic effect of the polypeptide fraction and eutypin was also studied with plasma membrane vesicles (Amborabé et al., 2001; Octave et al., 2006a). These toxins induced transmembrane potential variation and changes in transmembrane proton fluxes, and inhibited proton-coupled uptake of nutrients (Amborabé et al., 2001; Octave et al., 2006a). These experiments suggested that the polypeptide fraction alters proton flux both by inhibiting the plasma membrane proton-pumping activity and by increasing plasma membrane proton conductance (Octave et al., 2006a). However, the impact of the polypeptide fraction is not restricted to the plasma membrane since respiration and photosynthesis of grapevine leaf tissues were also inhibited by the polypeptide fraction (Octave et al., 2006a). Part of the toxin’s inhibitory effect is due to progressive reduction of the energetic charge of the cells by uncoupling and inhibition of photosynthesis and respiration (Amborabé et al., 2001). Therefore, a decreased energy charge may lead to dramatic metabolic starvation.
subsequent to decreased assimilate uptake in the cell. This may explain the dwarfed shoots and leaves observed on diseased plants (Octave et al., 2006a). Coordinated up-regulation of several genes involved in photosynthetic electron transport may help the cell to circumvent these effects at the chloroplast level. Although no such effect could be detected for the mitochondrial transporters, restoration of chloroplast function may provide enough energy to prevent the appearance of symptoms.

The present observations may also be related to a recent work of Valtaud et al. (2009) who showed that Esca, another major vascular disease of grapevine, modified glutathione metabolism in a systemic way. Glutathione is a major compound for maintenance of the redox balance. In the present work, the up-regulation of genes encoding proteins of the thylakoid electron transport chain, and of the chloroplast thioredoxin M-type (B9GTN8) suggests that the plant may efficiently prevent the appearance of eutypiosis symptoms by restoring chloroplast electron transport and redox balance. This is further confirmed by the up-regulation of three other genes involved in redox balance: peroxiredoxin (B9MT31), thioredoxin peroxidase (B3TLV1), and glutaredoxin (B9MYC1) (Table 4).

Conclusions

The response of grapevine to _E. lata_ was studied by microarray analysis with: (i) foliar material distant from the infection point; (ii) the susceptible cultivar Cabernet-Sauvignon; (iii) aggressive _E. lata_ strains BX1-10 and NE85-1; and (iv) at the symptom externalization time point. Although many genes involved in defence reactions are up-regulated in infected plants with symptoms, those genes do not seem efficient in preventing the detrimental effect of the fungus. Lack of symptoms is associated mainly with up-regulation of genes encoding proteins involved in photosynthetic electron transport and in the maintenance of redox balance. The data and these genes may give some clues about strategies aiming to prevent or to fight eutypiosis.

Acknowledgements

We thank the Comité Interprofessionnel du Vin de Bordeaux (CIVB) for the PhD grant allocated to CC and for partial funding of the research expenses. The authors also thank David Lafarge and Philippe Cartolaro (UMR Santé Végétale, INRA-ENITA Bordeaux, France) for providing leaves infected by _P. viticola_ and _E. necator_, Damien Afoufa-Bastien (FRE CNRS 3091, University of Poitiers, France) for providing leaf samples infected by _B. cinerea_, Jean Pierre Péros (UMR Diversité et génomes des plantes cultivées, INRA Montpellier, France) for providing _E. lata_ strains, Jean Michel Liminana (UMR Santé Végétale, INRA-ENITA Bordeaux, France) for his technical assistance during the production and characterization of greenhouse and vineyard material, and Chateau Cruzeaux (INRA Bordeaux) for the kind permission to collect vine samples. The authors also thank Yohann Petit (UMR Biologie du Fruit, INRA Bordeaux, France), Romain Fouquet, and Sabine Guillaumie (UMR EGTV, ISVV, INRA Bordeaux, France) for their help during microarray technical improvements. The authors would like to express their gratitude to Julie Scholes (Department of Animal and Plant Sciences, University of Sheffield, UK) for her critical reading and comments on the manuscript.

Supplementary data

Supplementary data are available at _JXB_ online.

**Table S1.** Sequences and melting temperatures of primers used for semi-quantitative RT-PCR of candidate genes selected after microarray analysis. The expected size of the amplified products is indicated in bp.

**Table S2.** Grapevine genome identifier (G8X ID), DFCI grape gene index version 6 identifier (VvGI6 ID), and the protein ID associated with the sequences differentially expressed between S+R+ and S−R− plants, for at least two conditions: _in vitro_ (I), greenhouse (G), vineyard (V).

**Table S3.** Grapevine genome identifier (G8X ID), DFCI grape gene index version 6 identifier (VvGI6 ID), and the protein ID of the sequences associated with absence of symptoms.

References

Amborabé BE, Fleurat-Lessard P, Bonmort J, Roustan JP, Roblin G. 2001. Effects of eutypine, a toxin from _Eutypa lata_, on plant cell plasma membrane: possible subsequent implication in disease development. Plant Physiology and Biochemistry 39, 51–58.

Aziz A, Heyraud A, Lambert B. 2004. Oligogalacturonide signal transduction, induction of defense-related responses and protection of grapevine against _Botrytis cinerea_. Planta 218, 767–774.

Aziz A, Poinsot B, Daire X, Adrian M, Bezier A, Lambert B, Joubert JM, Pugin A. 2003. Laminarin elicits defense responses in grapevine and induces protection against _Botrytis cinerea_ and _Plasmopara viticola_. Molecular Plant-Microbe Interactions 16, 1118–1128.

Belhadj A, Saigne C, Telef N, Cluzet S, Bouscaut J, Corio-Costet MF, Merillon JM. 2006. Methyl jasmonate induces defense responses in grapevine and triggers protection against _Erysiphe necator_. Journal of Agriculture and Food Chemistry 54, 9119–9125.

Bernard AC, Mur G. 1986. Les sites de présence sur la souche du mycélium d’_Eutypa lata_, agent causal de l’eutypiose de la Vigne. Progrès Agricole et Viticole 103, 288–289.

Bezier A, Lambert B, Baillieul F. 2002. Cloning of a grapevine _Botrytis_-responsive gene that has homology to the tobacco hypersensitivity-related _hsr203J_. Journal of Experimental Botany 53, 2279–2286.

Boubals D. 1986. L’eutypiose est actuellement la plus grave maladie de la vigne. Progrès en Agriculture et Viticulture 103, 358–360.

Carter MV. 1960. Further studies on _Eutypa armeniacae_. Australian Journal of Agricultural Research 11, 498–505.
Carter MV. 1965. Ascospore deposition of Eutypa armeniacae. Australian Journal of Agricultural Research 16, 825–836.

Catal M, Jordan SA, Butterworth SC, Schilder AMC. 2007. Detection of Eutypa lata and Eutypella vitis in grapevine by nested multiplex polymerase chain reaction. Phytopathology 97, 737–747.

Chapuis L. 1995. L’Eutypiose de la vigne: contribution à l’étude des relations hôte–parasite. PhD thesis, Université de Bordeaux 2, Bordeaux.

Chapuis L, Richard L, Dubos B. 1998. Variation in susceptibility of grapevine pruning wound to infection by Eutypa lata in south-western France. Plant Pathology 47, 463–472.

Creaser M, Wicks T. 2001. Yearly variation in Eutypa dieback symptoms and the relationship to grapevine yield. Australian and New Zealand Grapegrower and Winemaker 452, 50–52.

Darrieutort G, Lecomte P. 2007. Evaluation of a trunk injection technique to control grapevine wood diseases. Phytopathologia Mediterranea 46, 50–57.

Deswarte C, Eychenne J, Davy de Virville J, Roustan JP, Moreau F, Fallot J. 1996. Protonophoric activity of eutypine, a toxin from Eutypa lata, in plant mitochondria. Archives of Biochemistry and Biophysics 334, 200–205.

Deswarte C, Rouquier P, Roustan JP, Dargent R, Fallot J. 1994. Ultrastructural changes produced in plantlet leaves and protoplasts of Vitis vinifera cv. Cabernet Sauvignon by eutypine, a toxin from Eutypa lata. Vitis 33, 185–188.

Dowd C, Wilson LW, McFadden H. 2004. Gene expression profile changes in cotton root and hypocotyl tissues in response to infection with Fusarium oxysporum f. sp. vasinfectum. Molecular Plant-Microbe Interactions 17, 654–667.

Duthie JA, Munkvold GP, Marois JJ, Grant S, Chellemi DO. 1991. Relationship between age of vineyard and incidence of Eutypa dieback. Phytopathology 81, 1183.

Eighzaliz B, Gas G, Fallot J. 1992. Biodegradation of lignocelluloses in grapevine (Vitis vinifera cv. Cabernet Sauvignon) by Eutypa lata (Pers Fr) Tul. Vitis 31, 95–103.

English H, Davis JR. 1978. Eutypa armeniacae in apricot: pathogenesis and induction of xylem soft-rot. Hilgardia 46, 193–204.

Frick UB, Schaller A. 2002. cDNA microarray analysis of fusococcin-induced changes in gene expression in tomato plants. Planta 216, 83–94.

Fung RWM, Gonzalo M, Fekete C, Kovacs LG, He Y, Marsh E, McIntyre LM, Schachtman DP, Qiu WP. 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 RWM, Qiu WP, Su YC, Schachtman DP, Huppert K, Fekete C, Kovacs LG. 2007. Gene expression variation in grapevine species Vitis vinifera L. and Vitis aestivalis Michx. Genetic Resources and Crop Evolution 54, 1541–1553.

Hamelin RC, Bourassa M, Rail J, Dusabenagasan M, Jacobi V, Lafamme G. 2000. PCR detection of Gremmeniella abietina, the causal agent of Scleroderris canker of pine. Mycological Research 104, 527–532.

Jaillon O, Aury JM, Noel B, et al. 2007. The grapevine genome sequence suggests ancestral hexaploidization in major angiosperm phyla. Nature 449, 463–465.

Kim JH, Mahoney N, Chan KL, Molyneux RJ, Campbell BC. 2004. Secondary metabolites of the grapevine pathogen Eutypa lata inhibit mitochondrial respiration, based on the model bioassay using the yeast Saccharomyces cerevisiae. Current Microbiology 49, 282–287.

Kortekamp A. 2006. Expression analysis of defence-related genes in grapevine leaves after inoculation with a host and a non-host pathogen. Plant Physiology and Biochemistry 44, 58–67.

Larbart R. 2006. Contribution à l’étude des P450 impliqués dans la biosynthèse des furocoumarines. PhD thesis, Institut National Polytechnique de Lorraine, Vandoeuvre-lès-Nancy.

Lardner R, Stummer BE, Sosnowski MR, Scott ES. 2005. Molecular identification and detection of Eutypa lata in grapevine. Mycological Research 109/, 799–808.

Lecomte P, Péros JP, Blanchard D, Bastien N, Delyé C. 2000. PCR assays that identify the grapevine dieback fungus Eutypa lata. Applied and Environmental Microbiology 66, 4475–4480.

Lemoine S, Combes F, Servant N, Le Crom S. 2006. Goulphar: rapid access and expertise for standard two-color microarray normalization methods. BMC Bioinformatics 7, 467.

Mahoney N, Lardner R, Molyneux RJ, Scott ES, Smith LR, Schoch TK. 2003. Phenolic and heterocyclic metabolite profiles of the grapevine pathogen Eutypa lata. Phytochemistry 64, 475–484.

Mauro MC, Vaillant V, Tey-Ruhl P, Mathieu Y, Fallot J. 1988. In vitro study of the relationship between Vitis vinifera and Eutypa lata (Pers.: Fr.) Tul. I. Demonstration of toxic compounds secreted by the fungus. American Journal of Enology and Viticulture 39, 200–204.

Moller WJ, Kasimatis AN. 1978. Dieback of grapevine caused by Eutypa armeniacae. Plant Disease Reporter 62, 254–258.

Molyneux RJ, Mahoney N, Bayman P, Wong RY, Meyer K, Irelan N. 2002. Eutypa dieback in grapevines: differential production of acetylenic phenol metabolites by strains of Eutypa lata. Journal of Agriculture and Food Chemistry 50, 1393–1399.

Monteiro S, Barakat M, Picarra-Pereira MA, Teixeira AR, Ferreira RB. 2003. Osmotin and thaumatin from grape: a putative general defense mechanism against pathogenic fungi. Phytopathology 93, 1505–1512.

Moy P, Qutob D, Chapman BP, Atkinson I, Gijzen M. 2004. Patterns of gene expression upon infection of soybean plants by Phytophthora sojae. Molecular Plant-Microbe Interactions 17, 1051–1062.

Munkvold GP, Duthie JA, Marois JJ. 1994. Reductions in yield and vegetative growth of grapevines due to Eutypa dieback. Phytopathology 84, 186–192.

Munkvold GP, Marois JJ. 1995. Factors associated with variation in susceptibility of grapevine pruning wounds to infection by Eutypa lata. Phytopathology 85, 249–256.

Novikov E, Barillot E. 2007. Software package for automatic microarray image analysis (MAIA). Bioinformatics 23, 639–640.

Octave S, Amborabé BE, Fleurat-Lessard P, Bergès T, Roblin G. 2006a. Modifications of plant cell activities by proteic compounds
excreted by *Eutypa lata*, a vineyard fungal pathogen. *Physiologia Plantarum* **128**, 103–115.

**Octave S, Roblin G, Vachaud M, Fleurat-Lessard P.** 2006b. Polypeptidic metabolites secreted by the fungal pathogen *Eutypa lata* participate in *Vitis vinifera* L. to cell structure damage observed in Eutypa dieback. *Functional Plant Biology* **33**, 297–307.

**Pascoe I.** 1999. Grapevine trunk disease—black goo decline, esca, Eutypa dieback and others. *Australian Grapegrower and Winemaker* **429**, 27–28.

**Peros JP, Berger G.** 1994. A rapid method to assess the aggressiveness of *Eutypa lata* isolates and the susceptibility of grapevine cultivar to Eutypa dieback. *Agronomie* **14**, 515–523.

**Peros JP, Berger G.** 1999. Diversity within natural progenies of the grapevine dieback fungus *Eutypa lata*. *Current Genetics* **36**, 301–309.

**Petzoldt CH, Moller WJ, Sall MA.** 1981. Eutypa dieback of grapevine: seasonal differences in infection and duration in susceptibility of pruning wounds. *American Phytopathological Society* **71**, 540–543.

**Philippe I, Fallot J, Petitprez M, Dargent R.** 1993. Effets de l’eutypiose sur des feuilles de *Vitis vinifera* cv. Cabernet Sauvignon. *Vitis* **31**, 45–53.

**Reid KE, Olsson N, Schlosser J, Peng F, Lund ST.** 2006. An optimized grapevine RNA isolation procedure and statistical determination of reference genes for real-time RT-PCR during berry development. *BMC Plant Biology* **6**, 27.

**Robb J, Lee B, Nazer RN.** 2007. Gene suppression in a tolerant tomato–vascular pathogen interaction. *Planta* **226**, 299–309.

**Rolshausen PE, Greve LC, Labavitch JM, Mahoney NE, Molyneux RJ, Gubler WD.** 2008. Pathogenesis of *Eutypa lata* in grapevine: identification of virulence factors and biochemical characterization of cordon dieback. *Phytopathology* **98**, 222–229.

**Rolshausen PE, Trouillas F, Gubler WD.** 2004. Identification of *Eutypa lata* by PCR-RFLP. *Plant Disease* **88**, 925–929.

**Rotter A, Camps C, Lohse M, et al.** 2009. Gene expression profiling in susceptible interaction of grapevine with its fungal pathogen *Eutypa lata*: extending MapMan ontology for grapevine. *BMC Plant Biology* **9**, 104–128.

**Schmidt CS, Wolf GA, Lorenz D.** 1999. Production of extracellular hydrolytic enzymes by the grapevine dieback fungus *Eutypa lata*. *Journal of Plant Disease Protection* **106**, 1–11.

**Smith LR, Mahoney N, Molyneux RJ.** 2003. Synthesis and structure–phytotoxicity relationships of acetylenic phenols and chromene metabolites, and their analogues, from the grapevine pathogen *Eutypa lata*. *Journal of Natural Products* **66**, 169–176.

**Smyth GK.** 2004. Linear models and empirical Bayes methods for assessing differential expression in microarray experiments. *Statistical Applications in Genetics and Molecular Biology* **3**, Article 3.

**Sosnowski MR, Lardner R, Wicks TJ, Scott ES.** 2007. The influence of grapevine cultivar and isolate of *Eutypa lata* on wood and foliar symptoms. *Plant Disease* **91**, 924–931.

**Tey-Ruhl P, Philippe I, Renaud JM, Tsoupras G, De Angelis P, Fallot J, Tabacchi R.** 1991. Eutypine, a phytotoxin produced by *Eutypa lata*, the causal agent of a dying arm disease of grapevine. *Phytochemistry* **30**, 471–473.

**Valtaud C, Foyer CH, Fleurat-Lessard P, Bourbouloux A.** 2009. Systemic effects on leaf glutathione metabolism and defence protein expression caused by esca infection in grapevines. *Functional Plant Biology* **36**, 260–279.

**Wicks T, Davis K.** 1999. The effect of *Eutypa* on grapevine yield. *Australian Grapegrower and Winemaker Annual Technical Issue*, 15–16.

**Zhao JW, Wang JL, An LL, Doerge RW, Chen ZJ, Grau CR, Meng JL, Osborn TC.** 2007. Analysis of gene expression profiles in response to *Sclerotinia sclerotiorum* in *Brassica napus*. *Planta* **227**, 13–24.