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RESEARCH PAPER

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

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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 (Peros and Berger, 1994). There is no known resistant cultivar (Boubals, 1986; Mauro *et al.*, 1988; Munkvold and Marois, 1995; Peros 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; Deswartes *et al.*, 1994, 1996; Amborabé *et al.*, 2001; Kim *et al.*, 2004; Octave *et al.*, 2006*b*), grapevine responses to *E. lata* are still poorly described.

The present work describes a trancriptomic 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 *Plasmopra 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 Eut02 F3 (TGGTGGACGGGTAGGGTTAG) and Eut02 R2 (GGCCTTACCGAAATAGACCAA). This indirect and destructive PCR allowed a clear identication 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 5× green buffer (Promega), 2 mM $MgCl_2$, 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 0.5× 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 vineyard 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 (5000 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 ArrayEpress (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 (Jaillon *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 denaturated 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 (532 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 (532 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 characteristics, 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 candidates 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 than synthesized by Operon. Primer sequences and predicted product size are given in Supplementary Table S1 available at *JXB* online.

About 2 µg 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, $MgCl_2$, 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,

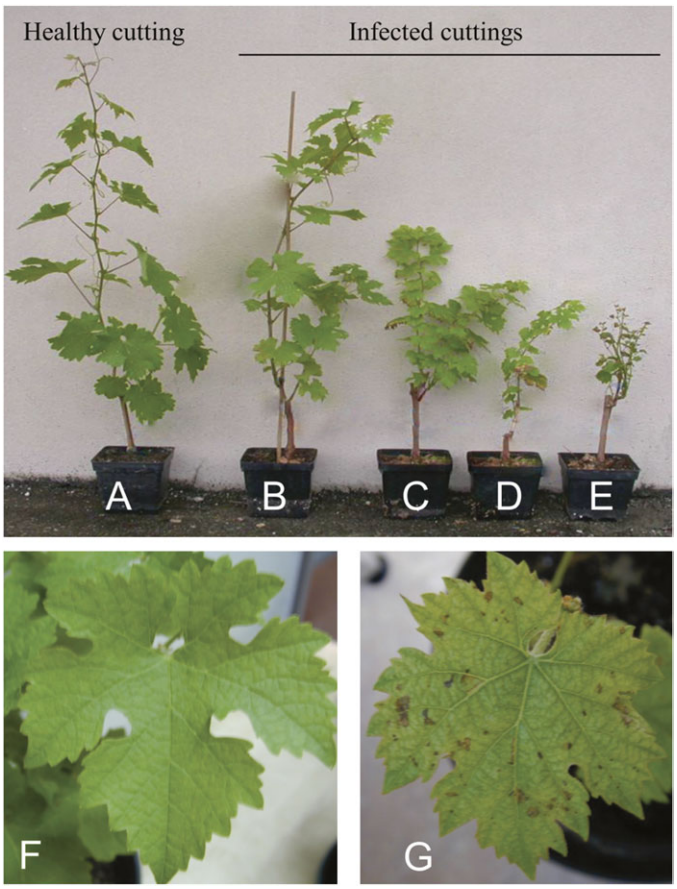

Fig. 1. Eutypiosis symptoms on Cabernet-Sauvignon greenhouse cuttings collected 1 year after experimental infection by the BX1-10 *E. lata* strain. (A) Control uninfected plant. (B–E) Infected plants exhibiting various degrees of symptoms. (F) Leaf of an uninfected plant. (G) Symptoms on a leaf from an infected plant.

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 re-isolation 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				
	2002	2003	2004	2005	2006	*E. lata* (rate/20)	*Botryosphaeria*	*P. chlamydospora*	*P. aleophilum*	*Trichoderma*
R18C38	**E2**	**BM E1**	**BM E1**	**BM E1/3**	**BM E1/3**	**20**	−	−	−	−
R16C19	**E1**	**S1**	**S2**	**E4**	**E4**	**17**	−	−	−	−
R8C45	**BM**	**BM**	**BM**	**BM E1**	**BM E1**	**12**	+	−	−	−
R10C39	**U E1**	**R S1**	**O**	**E1**	**U R E1**	**10**	+	−	−	−
R9C39	E4	E2 E4	O	E4	E2	8	+	−	−	−
R5C65	BM	BM	BM	BM S1/3	BM E1 E3	8	+	+	+	−
R18C56	BM	BM R	BM R	U R E2	UR E2/4	3	−	−	+	−
R6C23	BM	BM S1	BM	BM E3	BM E3 E1	2	−	−	−	+
R4C4	E1	E1	O	E1	E2	2	+	−	−	−
R13C66	BM E1	BM S1	BM	BM E1/3	BM E1/3	1	−	+	+	−
R11C20	BM	BM R	BM R	BM R E1	BM E1	1	+	+	+	−
R10C34	BM	BM E1	BM	BM R	BM E1	1	+	+	+	−
R18C12	***0***	***0***	***0***	***0***	***0***	***0***	+	−	−	−
R18C23	***0***	***0***	***0***	***0***	***0***	***0***	+	−	−	−
R13C8	***0***	***0***	***0***	***0***	***0***	***0***	+	−	−	−
R17C18	***0***	***0***	***0***	***0***	***0***	***0***	+	−	−	−
R12C47	0	0	0	0	0	0	+	+	+	−
R16C49	0	0	0	0	0	0	+	−	+	−
R17C22	0	0	0	0	0	0	−	−	−	+
R11C48	0	0	0	0	0	1	+	−	−	−
R8C60	0	0	0	0	0	2	−	−	−	−
R19C8	0	0	0	0	0	5	−	+	+	−
R4C68	0	0	0	0	0	6	+	+	−	−
R10C12	*0*	*0*	*0*	*0*	*0*	*9*	+	−	−	−
R18C9	*0*	*0*	*0*	*0*	*0*	*10*	−	−	−	−
R17C4	*0*	*0*	*0*	*0*	*0*	*12*	+	−	−	−
R20C3	*0*	*0*	*0*	*0*	*0*	*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.

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.

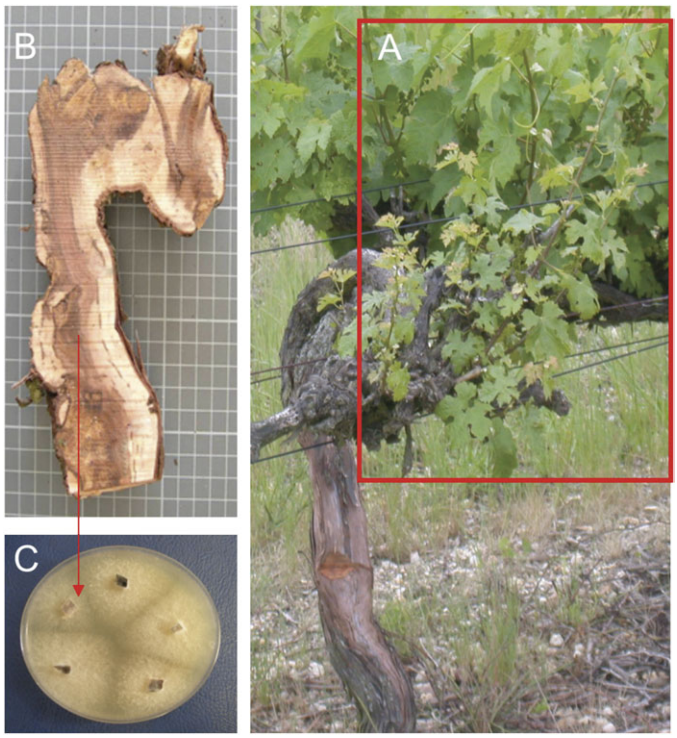

Fig. 2. Cabernet-Sauvignon grapevine naturally infected in the vineyard. (A) Leaf symptoms. (B) Typical sectorial necrosis from which *E. lata* mycelium may be re-isolated (C).

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).

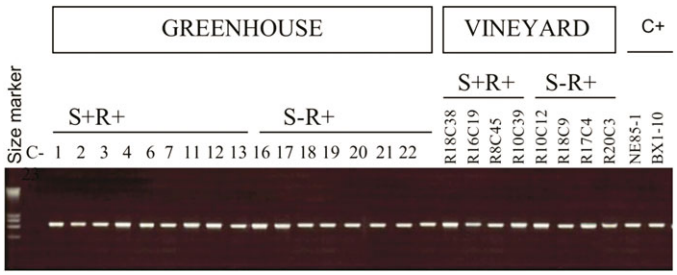

Fig. 3. Indirect PCR identification of the presence of *E. lata* in vineyard and greenhouse plants. The tested samples are mycelia growing from S^+R^+ and S^-R^+ greenhouse plants infected with the BX1-10 *E. lata* strain and from vineyard S^+R^+ and S^-R^+ plants. The PCR was also run either with DNA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

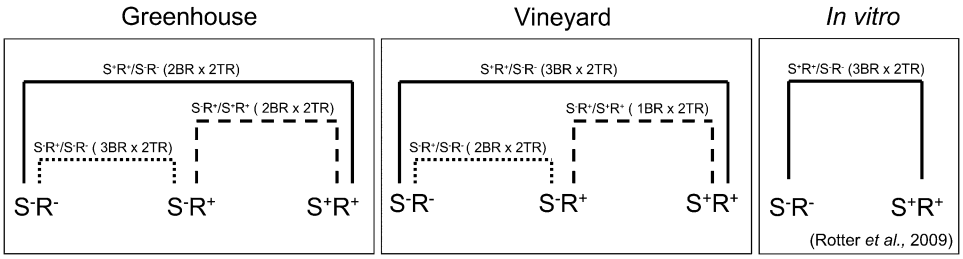

Fig. 4. Microarray experimental design. The microarray data produced with greenhouse and vineyard material described herein were combined with data that we obtained previously in *in vitro* conditions (Rotter *et al.*, 2009). Three kind of plants were characterized: infected with symptoms (S^+R^+), infected without symptoms (S^-R^+), and healthy (S^-R^-), and 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.

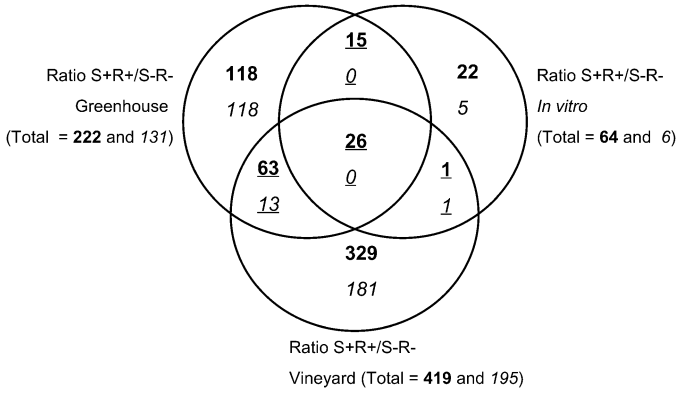

Fig. 5. Venn diagram showing the distribution of genes differentially expressed (*P*-value ≤0.05 and threshold ≥1.5) between infected plants with symptoms (S^+R^+) and healthy plants (S^-R^-) grown *in vitro*, in the greenhouse, and in the vineyard. The numbers of up- and down-regulated genes in infected 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.

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

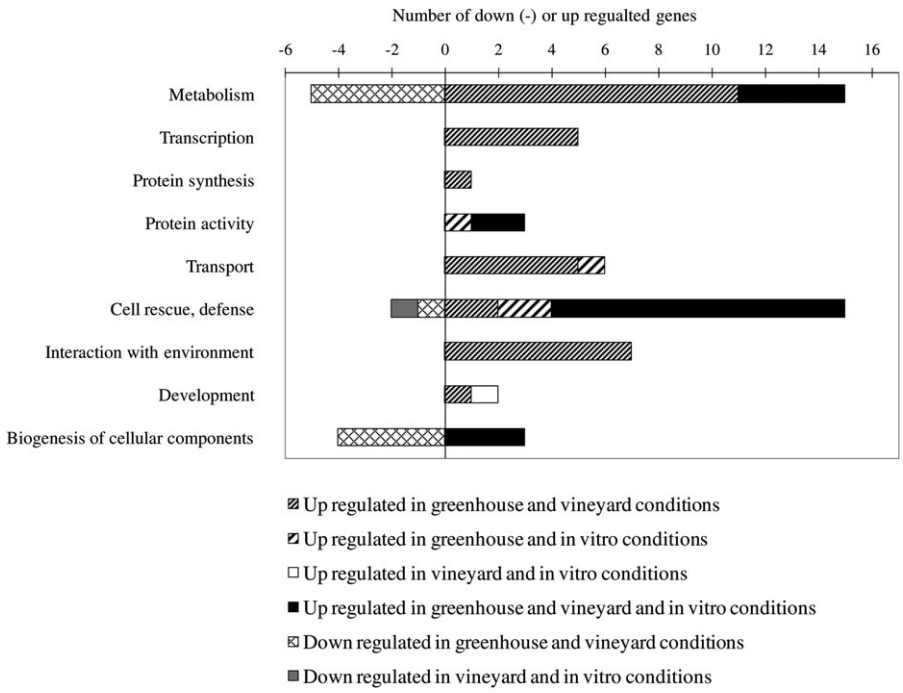

Fig. 6. Distribution into functional categories of genes differentially expressed between S^+R^+ and S^-R^- plants (*P*-value 0.05 and threshold 1.5) in at least two growth conditions. Only the genes showing a good homology with known genes were considered. The number of genes of each category is reported 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* and vineyards 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 ⩾1.5 or ⩽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		*In vitro* (I)		Greenhouse (G)		Vineyard (V)	
		Regulation	Condition	Ratio	*P*-value	Ratio	*P*-value	Ratio	*P*-value
Extracellular metabolism									
Vv_10002068	Homologue to β-1,3-glucanase complete	Up	I+G+V	1.864	3.95E-07	1.5568	0.00256	2.9927	2.6E-05
Vv_10000389	Similar to β-1,3-glucanase complete	Up	I+G+V	2.005	2.27E-07	1.5752	0.003344	1.7724	2.4E-07
Vv_10010418	Similar to β-1,3-glucanase complete	Up	I+G+V	2.057	5.79E-08	1.0505	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	1.047	0.314894	1.6817	0.004743	1.6935	2.4E-06
Vv_10001606	Similar to asparagine synthetase complete	Up	G+V	1.015	0.829175	2.848	0.007312	3.3318	4.2E-08
Vv_10004099	Similar to asparagine synthase-related protein complete	Up	G+V	0.655	2.83E-07	2.0526	0.001701	4.907	1.2E-09
Phenylpropanoid metabolism									
Vv_10004786	Similar to acyl:CoA ligase complete	Up	G+V	0.905	0.009552	1.6602	0.003996	1.6719	9.5E-06
Vv_10011235	Weakly similar to tetrahydroxychalcone 2′-glucosyltransferase complete	Up	G+V	0.962	0.52379	1.7886	0.001989	1.9321	9E-07
Vv_10002511	Weakly 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	1.028	0.510806	1.7368	0.001049	1.8316	1E-06
Vv_10000306	Weakly similar to β-amylase complete	Up	G+V	0.869	0.095964	2.1877	0.024777	1.8991	4.6E-07
Vv_10003056	Similar to putative fructokinase-5 complete	Down	G+V	0.978	0.600565	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	0.00123	0.4415	0.005116	0.6303	1.8E-06
Vv_10008537	Weakly similar to GDSL esterase/lipase partial (93%)	Down	G+V	0.97	0.654092	0.6076	0.007392	0.619	4.8E-06
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	0.736553	1.8856	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	0.00147	0.5284	0.002599	0.6409	3.2E-05
Vv_10001696	Weakly similar to fasciclin-like AGP 11 partial (63%)	Down	G+V	1.256	0.000394	0.5096	0.002	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_10004211	Similar to xyloglucan endotransglycosylase partial (96%)	Down	G+V	0.872	0.001466	0.4261	0.00048	0.4719	9.3E-08
Vv_10011060	Weakly similar to HyPRP2 partial (84%)	Up	G+V	–	–	3.9942	1.62E-05	3.3677	6.3E-08
Vv_10011061	Weakly similar to HyPRP2 partial (84%)	Up	G+V	–	–	4.6476	0.000207	2.7843	3.1E-07
Vv_10010712	Similar to XET complete	Up	G+V	1.017	0.803459	1.669	0.005678	1.8456	0.0233
Defence response									
Vv_10008543	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_10003617	Similar to osmotin-like protein complete	Up	I+G+V	1.848	0.000371	2.9414	0.000341	2.073	0.00039
Vv_10010885	Homologue to osmotin-like protein complete	Up	I+G+V	2.824	3.05E-08	6.9775	8.96E-05	3.2301	1E-06
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

Table 3. *Continued*

Probe ID	Annotation	Profile		*In vitro* (I)		Greenhouse (G)		Vineyard (V)	
		Regulation	Condition	Ratio	*P*-value	Ratio	*P*-value	Ratio	*P*-value
Vv_10010887	Homologue to pathogenesis-related protein 10.3 partial (58%)	Up	I+G+V	1.801	0.005897	2.8363	0.000641	3.0526	2.8E-06
Vv_10011243	Homologue to putative pathogenesis-related protein 1 partial (88%)	Up	I+ G+V	2.372	2.3E-06	1.8816	0.008447	1.7636	0.00022
Vv_10004981	Similar to putative pathogenesis-related protein 1 partial (88%)	Up	I+G+V	2.507	1.43E-05	1.6573	0.003028	1.6072	5.3E-05
Vv_10000483	Similar to NtPRp27 partial (89%)	Up	G+V	1.381	0.002058	2.001	0.000373	1.5147	0.00048
Vv_10009597	Weakly similar to hairpin-inducing protein complete	Up	I+G+V	2.367	1.29E-07	5.3487	0.000544	4.3763	6.1E-08
Vv_10000872	Similar to rhaumatin-like protein partial (93%)	Up	I+G	1.585	8.88E-08	2.8329	0.00054	1.3791	5.9E-05
Vv_10000136	Homologue to class IV chitinase partial (92%)	Up	I+G+V	2.677	2.79E-08	7.4383	0.000282	7.6528	1.2E-10
Vv_10002903	Similar to class IV chitinase partial (94%)	Up	I+G+V	2.18	4.5E-08	2.3405	0.001381	1.5841	1.7E-05
Vv_10004018	Similar to class IV chitinase complete	Up	I+G	1.514	4.81E-05	1.6071	0.021867	1.3173	0.00033
Vv_10000957	Weakly similar to glutathione *S*-transferase GST 18 complete	Up	G+V	1.144	0.001318	1.5802	0.010604	1.815	3.6E-07
Vv_10008745	Weakly similar to peroxidase partial (94%)	Up	I+G+V	1.572	4.72E-07	2.6628	8.02E-05	2.5459	9.8E-09
Vv_10004303	Similar to glutaredoxin complete	Down	G+V	0.984	0.70024	0.6376	0.01423	0.6474	1.2E-05
Vv_10010268	Weakly similar to disease resistance response protein partial (84%)	Up	I+G+V	3.518	1.82E-06	8.0912	4.37E-06	2.6561	1.9E-08
Protein activity									
Vv_10011266	Similar to tumour-related protein partial (89%)	Up	I+G+V	3.796	4.8E-10	5.5458	6.58E-05	6.3066	1.1E-07
Vv_10001691	Similar to tumour-related protein partial (89%)	Up	I+G+V	4.586	1.54E-11	14.975	2.69E-06	9.3738	6.1E-08
Vv_10006852	Weakly similar to inhibitor of trypsin and hageman factor complete	Up	I+G	1.774	0.011188	6.5979	0.00014	1.4981	0.03772
Transcription									
Vv_10008748	Weakly similar to AP2/ERF transcription factor partial (85%)	Up	G+V	1.053	0.190985	1.528	0.002243	2.2689	6.2E-08
Vv_10001736	Weakly similar to WRKY transcription factor-b partial (89%)	Up	G+V	0.988	0.673598	2.6682	8.65E-05	2.3055	2.3E-06
Vv_10001880	Homologue to putative WRKY transcription factor 30 partial (94%)	Up	G+V	0.905	0.010825	1.6407	0.001981	1.5068	1.4E-06
Vv_10004421	Weakly similar to zinc-finger protein 1 complete	Up	G+V	0.976	0.392951	1.5908	0.001671	1.7336	3.9E-05
Vv_10004205	Weakly similar to NAC domain protein NAC4 complete	Up	G+V	0.986	0.739087	2.0518	0.000432	1.7835	1.8E-07
Transport									
Vv_10010759	Homologue to aquaporin partial (95%)	Up	G+V	1.261	0.000189	1.8718	0.003966	2.1035	0.00186
Vv_10014047	Weakly similar to amino acid transporter (fragment) complete	Up	G+V	1.095	0.369001	2.1475	0.000616	1.5091	0.01053
Vv_10004892	Weakly similar to metal ion-binding protein complete	Up	G+V	1.487	6.67E-05	1.6271	0.001291	2.096	7.9E-06
Vv_10000751	Weakly similar to heavy metal transport/detoxification protein complete	Up	G+V	1.049	0.350084	1.8975	0.044426	2.0149	1E-06
Vv_10009149	Weakly similar to exocyst protein partial (92%)	Up	G+V	1.109	0.028727	1.6735	0.001875	1.9561	1.7E-06
Vv_10003601	Similar to glucose-6-phosphate translocator partial (82%)	Up	I+G	1.556	5.68E-05	1.5412	0.033235	0.968	0.34449
Interaction with environmemt									
Vv_10011427	Weakly similar to 1-aminocyclopropane-1-carboxylate oxidase complete	Up	G+V	1.221	0.000255	1.8147	0.000649	1.7062	2.6E-06
Vv_10004370	Similar to 1-aminocyclopropane-1-carboxylate oxidase 3 complete	Up	G+V	1.192	0.082893	2.0799	0.000779	2.6507	1.6E-07
Vv_10001785	Similar to nitrilase 4B partial (92%)	Up	G+V	1.424	1.19E-06	2.2081	0.005411	2.3886	1.1E-07
Vv_10000183	Weakly similar to putative auxin-repressed complete	Up	G+V	0.79	0.005281	2.5562	0.000748	1.7584	2E-07
Vv_10001211	Weakly similar to auxin-responsive protein IAA26 partial (86%)	Up	G+V	0.954	0.505271	1.5304	0.00823	1.9773	6.5E-06
Vv_10013495	Similar to GID1-2 complete	Up	G+V	0.974	0.487208	1.6397	0.002657	2.3734	3.9E-07
Vv_10003687	Weakly similar to early light-inducible protein complete	Up	G+V	0.936	0.127152	1.5169	0.003052	1.8717	7.9E-06
Protein synthesis									
Vv_10000746	Weakly similar to deacetoxyvindoline 4-hydroxylase partial (92%)	Up	G+V	1.235	5.99E-05	1.9631	0.001953	1.775	8.8E-07
Development									
Vv_10000694	Weakly similar to senescence-associated partial (92%)	Up	G+V	0.853	0.001296	1.858	0.023539	1.9233	3.4E-07
Vv_10011267	Weakly similar to phytosulphokine-β partial (57%)	Up	I+G	1.58	1.33E-06	1.8682	0.005752	0.6719	2.5E-05

stems. For this reason, and to make sure any gene that may be differentially expressed was not missed, thresholds of 1.5 for up-regulation and 0.66 for down-regulation, with a *P*-value <0.05, were used. With a threshold of 1.5 for up-regulation and 0.66 for down-regulation, and a *P*-value <0.05 the numbers of overexpressed or down-regulated genes in S^+R^+ plants compared with S^-R^- plants were 64, 222, and 420, and 6, 131, and 195, respectively, under *in vitro*, greenhouse, and vineyard conditions. Venn diagrams were constructed to identify genes that exhibited the same behaviour for *in vitro*, greenhouse, and vineyard plants (Fig. 5). Twenty-six genes were overexpressed in *in vitro*, greenhouse, and vineyard S^+R^+ plants compared with the corresponding S^-R^- plants. No down-regulated genes were found in common between *in vitro*, greenhouse, and vineyard plants. Sixty-three genes were up-regulated both in S^+R^+ greenhouse and vineyard plants compared with the corresponding healthy plants, and 13 down-regulated genes were found both in greenhouse and vineyard plants with symptoms (S^+R^+) compared with healthy plants. In *in vitro* and greenhouse conditions, 15 common genes were up-regulated in S^+R^+ plants. Only two differentially expressed genes (one up- and one down-regulated) were shared between *in vitro* and vineyard conditions (Fig. 5). A total of 105 genes were up-regulated for at least two conditions in S^+R^+ compared with S^-R^- plants, and a total of 14 genes were down-regulated for at least two conditions. The number of up-regulated genes was thus much higher than the number of down-regulated genes.

Among the 119 genes which were differentially expressed in S^+R^+ plants for at least two conditions, 68 (57 up-regulated and 11 down-regulated) can be identified by mapping the probes to the *Vitis vinifera* Gene Index (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb = grape) or the Pinot noir grapevine genome (Jaillon at *al.*, 2007) and show a good homology with known genes. Classification of these genes in functional categories indicates that 12 categories were represented in overexpressed genes, whereas underexpression concerns only three categories (Fig. 6). Five categories are abundant for overexpressed genes: metabolism, defence reactions, interaction with the environment, transport, and transcription. Repressed genes belong to lipid metabolism, cell wall metabolism, and defence reactions.

The complete list of genes that were differentially affected is given in Table 3. Genes involved in carbon metabolism, amino acid, or phenylpropanoid metabolism were up-regulated in symptomatic infected plants. In contrast, several genes involved in lipid metabolism were down-regulated in these plants. Genes that are involved in defence reactions were quite numerous and most of them were up-regulated in infected plants with eutypiosis symptoms. They include osmotin, PR10 and PR1, arachidonic acid-induced DEA 1, harpin-induced protein Hin1, class IV chitinase and endochitinase, thaumatin, disease resistance proteins, and anionic peroxidase. Several genes encoding enzymes of cell wall metabolism or extracellular metabolism were also up-regulated. These include proline-rich protein, hydroxyproline-rich glycoprotein, and β-glucanase. In contrast, a few genes involved in plant cell wall metabolism were down-regulated, including those encoding an arabinogalactan, an expansin, a xyloglucan endotransglycosylase, a pectin methylesterase inhibitor, and a germin-like protein. Several genes involved in the interaction with the environment were up-regulated. They are particularly associated with hormonal metabolism and response. These genes include those encoding enzymes of the ethylene biosynthetic pathway (ACC oxidase), auxin-repressed proteins, and a gibberellin receptor. Other up-regulated genes encode transcription factors (dehydration-responsive element-binding protein, WRKY, Zn-finger, and NAC transcription factors) and protein regulating factors (tumour-related protein and serine/threonine kinase). One plasma membrane aquaporin and several ion and metabolite transporters are up-regulated in infected plants.

Identification of genes associated with lack of symptoms

In order to identify genes that may prevent symptom development, comparisons were made (i) between infected plants without or with eutypiosis symptoms (S^-R^+/S^+R^+) and (ii) between infected plants without symptoms and healthy plants (S^-R^+/S^-R^-). Because both types of plants are infected by *E. lata*, the first comparison (S^-R^+/S^+R^+) identifies genes that prevent symptom development and genes associated with symptom externalization. The second comparison (S^-R^+/S^-R^-) identifies genes that prevent symptom development and genes associated with response to infection by *E. lata*. Genes that prevent symptom development (even though the fungus is present in the plant) must be common between both comparisons (S^-R^+/S^+R^+) and (S^-R^+/S^-R^-). A total of 32 and 59 genes specifically involved in the absence of symptoms have been highlighted in greenhouse and vineyard conditions, respectively. Expression ratios obtained for the three comparisons (S^+R^+/S^-R^-, S^-R^+/S^+R^+, and S^-R^+/S^-R^-) allow 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 *b*6, 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^-	
		Regulation	Condition	Ratio	*P*-value	Ratio	*P*-value	Ratio	*P*-value
Lipid metabolism									
Vv_10009444	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_10003778	Similar to anthocyanidin synthase complete	Up	V	1.790	0.00481	1.560	0.00927	0.749	0.00062
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
Carbon 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	0.735	0.00294
Vv_10000154	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	0.15126
Amino acid metabolism									
Vv_10000953	Glutamine synthetase partial (97%)	Up	V	1.752	0.00512	1.459	0.01092	0.8438	0.00858
Energy photosyntesis									
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	0.00322	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	0.00038	1.048	0.54933
Vv_10000222	Similar to oxygen-evolving enhancer protein 2 complete	Up	V	1.627	0.01653	1.443	0.02712	0.734	0.00581
Vv_10000172	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	0.40738
Vv_10008623	Weakly similar to thylakoid lumenal 16.5 kDa protein partial (68%)	Up	V	1.800	0.01531	1.469	0.04659	0.740	0.00061
Vv_10004046	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_10003838	Homologue to phosphoribulokinase complete	Up	V	1.683	0.00582	1.422	0.01665	0.749	0.00087
Vv_10004505	ABC-ATPase, partial (89%)	Up	V	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_10004810	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_10002247	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

Table 4. *Continued*

Probe ID	Annotation	Profile		S^-R^+/S^+R^+		S^-R^+/S^-R^-		S^+R^+/S^-R^-	
		Regulation	Condition	Ratio	*P*-value	Ratio	*P*-value	Ratio	*P*-value
Signal transduction									
Vv_10009335	Similar to SNF1-related protein kinase regulatory β subunit 1 complete	Up	G	1.491	0.00388	1.522	0.00086	1.220	0.08501
Defence response									
Vv_10000593	Homologue to alcohol dehydrogenase complete	Up	V	0.651	0.01011	0.666	0.04479	1.3975	0.00002
Vv_10000137	Similar to class IV chitinase partial (94%)	Down	G	0.520	0.01489	0.518	0.00023	1.07946	0.75898
Vv_10004632	Similar to peroxiredoxin complete	Up	V	1.891	0.00293	1.558	0.00936	0.85771	0.03281
Vv_10004032	Similar to thioredoxin peroxidase partial (97%)	Up	V	1.585	0.01981	1.492	0.01098	0.82073	0.00042
Vv_10001459	Weakly similar to glutaredoxin complete	Up	G	1.450	0.00483	1.740	0.00002	1.26428	0.03457
Interaction with environment									
Vv_10004355	Weakly similar to wound-induced protein (fragment) partial complete	Down	G	0.441	0.00013	0.589	0.00006	1.17891	0.18634
Biogenesis of cellular compounds: cell wall									
Vv_10008631	β-Galactosidase complete	Down	V	0.645	0.01026	0.618	0.00378	1.24747	0.00067
Vv_10004343	Similar to expansin-like protein (fragment) partial (94%)	Up	G	1.506	0.00207	1.614	0.00005	1.35909	0.00848
Vv_10000080	Weakly similar to HyPRP2 partial (61%)	Down	G	0.271	0.00018	0.390	0.00002	1.36504	0.09341
No classification									
Vv_10009331	Weakly similar to Fw2.2 partial (83%)	Up	G	1.542	0.00887	1.410	0.00072	1.03609	0.83586
Vv_10008923	Similar to At1g64680 complete	Up	G+V	1.734	0.01552	1.418	0.01871	0.79571	0.00007

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

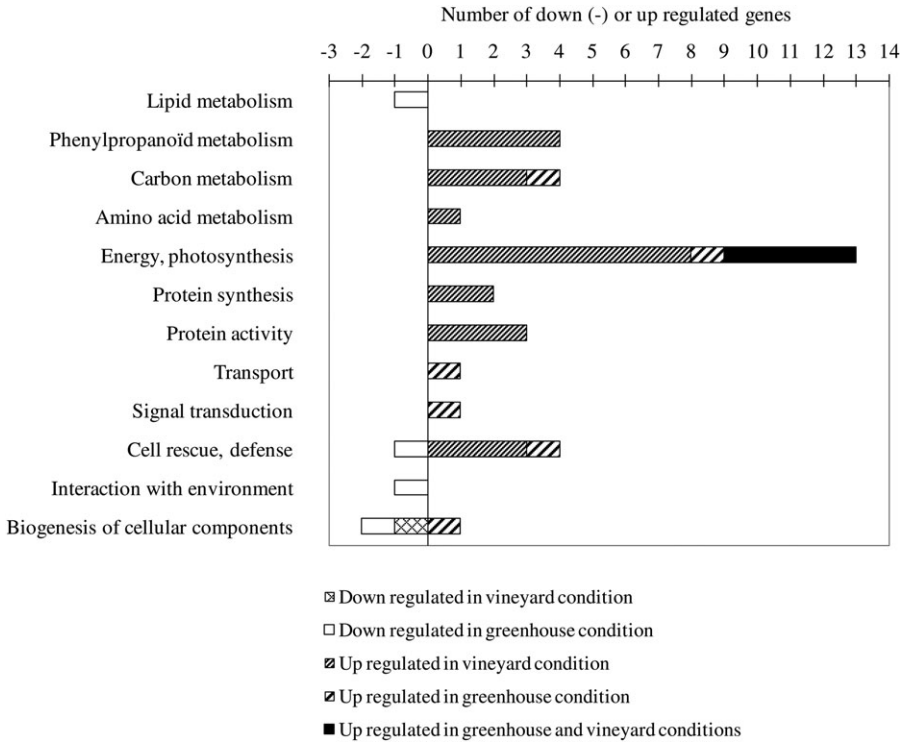

Fig. 7. Distribution into functional categories of differentially expressed genes which are associated with lack of symptoms. These genes are differentially expressed (threshold ⩾1.4, *P*-value ⩽0.05) in greenhouse and vineyard plants, and common to S^-R^-/S^+R^+ and S^-R^+/S^+R^+ comparisons between 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.

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.

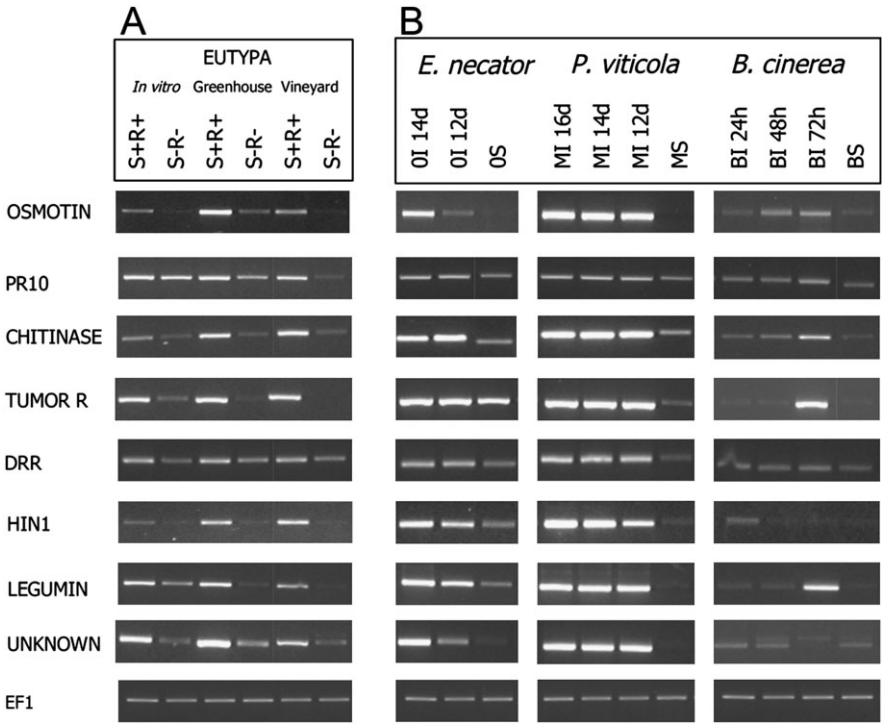

Fig. 8. RT-PCR expression analysis for candidate genes selected from the microarray analysis. The genes selected are all up-regulated in S^+R^+ plants compared with S^-R^- plants, for all the three types of conditions tested. (A) Response to *E. lata*. The expression was studied with the same plants 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*.

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

GSVIVG00027001001 (Vv_10001880) are associated with the transcription factors CaWRKY-b (AY743433) and VaWRKY 30 (AY509152). Both these transcription factors are overexpressed in *V. vinifera* leaves of a susceptible cultivar infected with *E. necator* compared with healthy grapevine leaves (Fung *et al.*, 2007). Both GSVIVG00001107001 (Vv_10003617) and GSVIVG00001106001 (Vv_10010885) are highly homologous to a *V. vinifera* gene encoding an osmotin (P93621). This protein has a strong antifungal activity *in vitro* 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 grapevine 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: GSVIVG00034623001) encoding a class IV chitinase (Q7XB39), *VvPIN* (Vv_10008543: GSVIVG00029889001) encoding a protease inhibitor (Q6YEY6), and the gene (Vv_10010418: 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_10002068) 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 strenghten the plant cell wall (anionic peroxidase, proline-rich and hydroxyproline-rich proteins), to maintain the osmotic balance (osmotin, DEA1), to destroy the fungal cell walls (chitinase, endo-chitinase, β-glucanase), and react to pathogen infection (PR). Induction of genes of secondary metabolism (PAL, flavanone-3-hydroxylase) and of aquaporins, ions, and metabolite transporters also follows these trends. In the present experiments, all those genes were unable to prevent infection and appearance of symptoms, because they are expressed too late, and/or at too low level, and/or are not appropriate. In order to identify tolerance/resistance genes, it will be interesting to compare results obtained here (in a susceptible cultivar) and other microarray analyses conducted with a more resistant cultivar.

The expression profile of selected genes obtained by RT-PCR confirmed the microarray expression profile (Fig. 8). These genes were up-regulated in S^+R^+ compared with S^-R^- plants in all the conditions tested. They were also up-regulated in Cabernet-Sauvignon leaves infected by *E. necator*, *P. viticola*, and *B. cinerea* (Fig. 8). This result was expected for genes involved in general defence mechanisms such as osmotin, PR10, chitinase, tumour-related protein, and legumin. The RT-PCR profiles obtained for some genes are in agreement with literature data. Thus, the GSVIVG00001106001 (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^+/S^+R^+ and S^-R^-/S^-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.*, 2006*a*). 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.*, 2006*a*). 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.*, 2006*a*). 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.*, 2006*a*). 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.*, 2006*a*). 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.*, 2006*a*). 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.

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.

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