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Identification of grapevine marker genes for early, non-destructive *Eutypa lata* infection diagnosis

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Eutypa lata is the causal agent of eutypa dieback, a highly damaging trunk disease affecting all grape-growing areas, with currently neither an efficient curative treatment nor an early non-destructive diagnostic method. The present work was carried out to discover grapevine genes expressed in response to the presence of *E. lata* that could be useful to develop an early (before visible foliar symptoms) and non-destructive (using grapevine leaves) diagnostic tool. Microarray analyses were carried out from (i) infected plants showing characteristic *E. lata* foliar and vascular symptoms and positive pathogen recovery from vascular lesions (S^+R^+), (ii) infected plants showing no symptoms (S^-R^+), and (iii) symptomless plants with negative pathogen recovery (S^-R^-). Vineyard and greenhouse-grown plants, naturally or artificially infected respectively, and uninoculated controls were characterized and leaf RNA was hybridized with 15k operon grapevine oligonucleotide microarrays. Among the grapevine genes differentially expressed between S^-R^+ and S^-R^- plants in greenhouse and vineyard conditions, 10 were highlighted as robust candidate genes for diagnosis: seven were specifically involved in response to infection and three were associated with symptom absence. Five were confirmed to be effective diagnostic marker genes usable in a qRT-PCR-based test performed on RNA extracted from grapevine leaves cultivated in either greenhouse or vineyard conditions. Furthermore, their expression profiles in response to infection with *E. lata* or other major grapevine fungi (*Erysiphe necator*, *Plasmopara viticola*, *Botrytis cinerea*) could be distinguished. The usefulness of these genes to develop an early and non-destructive method for diagnosis of *E. lata* infection is discussed with regard to the advantages and drawbacks of previous *E. lata* diagnostic studies.

Keywords: eutypa dieback, fungi, marker genes, microarray, *Vitis vinifera*

Introduction

Eutypa lata is an ascomycete fungus that causes a major grapevine trunk disease named eutypa dieback, also known as dying arm disease (Moller & Kasimatis, 1978; Carter, 1991). *Eutypa lata* infects at least 88 woody plant species in 52 genera and 27 families (Bolay & Carter, 1985), but grapevine is its primary target. Eutypa dieback has been reported in all grape-growing areas around the world (Carter, 1991). This disease has a significant economic impact on viticulture, mostly as a consequence of decreased yield (Carter, 1991; Wicks & Davis, 1999). It also reduces longevity of the grapevines (Munkvold & Marois, 1994) and increases vineyard management cost (Siebert, 2001). *Vitis vinifera* cultivars show significant differences in their susceptibility to *E. lata*, with Cabernet Sauvignon being particularly susceptible. No cultivar is known to be immune to infection (Peros & Berger, 1994; Sosnowski *et al.*, 2007). After initial infection, a common lag phase of 6–8 years is often observed before the appearance of first symptoms (Carter, 1991). Hence, by the time foliar symptoms are visible, the pathogen may have spread extensively throughout the vine. Symptoms of the disease include stunting of spring shoots, with small, cupped, chlorotic and tattered leaves, reduced

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development of fruit clusters, and characteristic dark, wedge-shaped necrosis of the wood in the trunk and cordons (Moller & Kasimatis, 1978; Carter, 1991). There is considerable annual variation in symptom expression, with vines commonly displaying symptoms one year but not in the subsequent year (Creaser & Wicks, 2001). Foliar symptoms have been attributed to toxins (Tey-Ruhl *et al.*, 1991; Mahoney *et al.*, 2005) and wall degradation enzymes (Rolshausen *et al.*, 2008). These compounds are produced by the fungus in the wood and are believed to be translocated to the shoots (Tey-Ruhl *et al.*, 1991), although no evidence has been reported to support this (Mahoney *et al.*, 2005).

There is no curative control for eutypa dieback and in the case of infection, if invaded wood is not removed, entire vines eventually die (Munkvold *et al.*, 1993). The removal of diseased tissue involves cutting off the trunk or the cordons at least 10–20 cm beyond the staining (Sosnowski *et al.*, 2007). Then, a healthy shoot can be selected and trained to rework a new vine or cordon. In the absence of curative treatment, avoiding or reducing infections can achieve control. Protecting the wounds or delaying the pruning period are common recommended practices. However, late pruning is not possible in all vineyards and, because growers rarely treat the pruning wounds more than once, these measures may lead to little reduction in disease (Weber *et al.*, 2007).

Several studies have been conducted in order to develop methods for diagnosis of *E. lata*. They are based on the *in planta* detection of the fungus by PCR tests (Lecomte *et al.*, 2000; Rolshausen *et al.*, 2004; Lardner *et al.*, 2005; Pilotti *et al.*, 2005), antibody detection (Octave *et al.*, 2009), or the *in planta* detection of compounds secreted by the fungus (Mahoney *et al.*, 2005; Lardner *et al.*, 2006; Rolshausen *et al.*, 2008). All these methods are destructive (they require trunk or cordon samples), labour-intensive and time-consuming.

In the present work, a different detection strategy was investigated. Microarrays were used to detect grapevine genes differentially expressed in leaves in response to the presence of *E. lata* in the trunk, even in the absence of foliar symptoms, and which could potentially be useful to develop an early, non-destructive diagnostic tool. The aims of this study, were (i) to identify eutypa dieback diagnostic marker genes by comparing the transcriptome of infected plants showing no symptoms (S^-R^+) and symptomless plants with negative pathogen recovery (S^-R^-), (ii) to use transcriptome comparisons to detect strong diagnostic markers more specifically associated with either the absence of symptoms or the response to infection, and (iii) to determine by RT-PCR and qRT-PCR the expression profile of candidate marker genes in response to infection by *E. lata* and other major grapevine fungi (*Erysiphe necator*, *Plasmopara viticola*, *Botrytis cinerea*).

Materials and methods

General strategy and experimental design

Data from a previous study (Camps *et al.*, 2010) were reanalysed in order to identify grapevine genes that might be used to develop an early and non-destructive *E. lata* diagnostic tool. To this end, genes were identified that were differentially expressed in the comparison (S^-R^+/S^-R^-) in greenhouse and vineyard conditions. Strong diagnostic markers associated with symptom absence were detected: these genes were differentially expressed in greenhouse and vineyard conditions, in both comparisons (S^-R^+/S^+R^+) and (S^-R^+/S^-R^-). Strong diagnostic markers associated with response to infection were also detected: these genes were differentially expressed in greenhouse and vineyard conditions, in both comparisons (S^+R^+/S^-R^-) and (S^-R^+/S^-R^-) (Fig. 1).

Infection and characterization of grapevine by *E. lata*

Samples were inoculated and characterized (symptom notation, *E. lata* recovery, PCR identification) as described in Camps *et al.* (2010). Briefly, infected and apparently uninfected Cabernet Sauvignon grapevines were obtained from the vineyard (natural infection). In the greenhouse, experimentally infected vines were compared with uninoculated plants. *Eutypa lata* foliar symptoms were evaluated for each grapevine 1 year after artificial infection with mycelia suspension in the greenhouse and between 2002 and 2006 in the vineyard. Foliar symptoms were categorized as not visible (S^-) or visible (S^+). For each grapevine, foliar material was collected after the final symptom evaluation, identified and stored at −80°C; meanwhile, presence of *E. lata* in the woody part was tested by fungal recovery and PCR identification. For both vineyard and greenhouse plants, cross-sections were made of the woody parts to look for brown lesions characteristic of eutypa dieback canker as described by Lecomte *et al.* (2000). In addition, wood fragments were sampled along the margin of the *E. lata* lesion (between healthy and infected wood). These segments were then split into wood chips

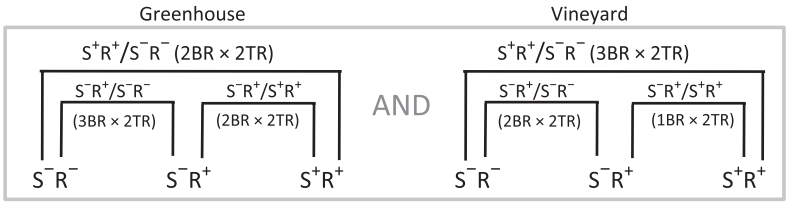

Figure 1 Experimental design for microarray analysis. Three kinds of plants were used: infected with symptoms (S^+R^+), infected without symptoms (S^-R^+) and symptomless plants with negative pathogen recovery (S^-R^-). Plant material was sampled from the vineyard and the greenhouse. Three comparisons of gene expression were performed: (S^+R^+/S^-R^-), (S^-R^+/S^-R^-), (S^-R^+/S^+R^+). For each comparison, the number of biological replicates (BR) and the number of technical replicates corresponding to the dye swap between cyanine 5 and cyanine 3 (TR) is specified.

(3 × 5 × 5 mm), and about 50 wood chips per grapevine were randomly selected, surface-sterilized by soaking in 3% calcium hypochlorite solution and placed onto Petri dishes containing malt (15 g L^{-1}) agar (20 g L^{-1}) medium supplemented with chloramphenicol (50 mg L^{-1}) for culture of *E. lata*. Petri plates for both greenhouse and vineyard samples were assessed visually for the presence of *E. lata* after 10 days of incubation in darkness at 22°C. PCR identification of *E. lata* was carried out as described previously (Lardner *et al.*, 2005) using SCAR primers Eut02 F3 (3′-TGGTGGACGGGTAGGGTTAG-5′) and Eut02 R2 (3′-GGCCTTACCGAAATAGACCAA-5′). Samples with positive recovery of *E. lata* and positive PCR were categorized as R^+ whereas samples were rated R^- in the case of negative isolation.

Infection of detached leaves with *Plasmopara viticola*, *Erysiphe necator* and *Botrytis cinerea*

In order to determine whether key changes in gene expression in leaves infected with *E. lata* (identified by global transcriptomic studies) were specific to this pathogen, they were also profiled by semiquantitative RT-PCR in grapevine leaves infected with other fungal pathogens, as described in Camps *et al.* (2010).

Microarray data production and analysis

As described in Camps *et al.* (2010), RNA was extracted from leaves of greenhouse and vineyard plants (S^+R^+, S^-R^+, S^-R^-) and amplified using the Amino Allyl MessageAmp II aRNA Amplification Kit (Ambion). Cy3 and Cy5 aRNA targets were hybridized to oligonucleotide microarrays allowing simultaneously monitoring of the expression of 14 562 grapevine transcripts. Microarray data is available under the accession number E-MEXP-2337 in ArrayExpress (http://www.ebi.ac.uk/arrayexpress).

The microarrays were scanned using GenePix v. 4.0 image acquisition software, quantified with the MAIA tool v. 2.75, and normalized with a modified version of the GOULPHAR script v. 1.1.2 (Lemoine *et al.*, 2006). 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 $\leq 0{\cdot}05$ and an expression ratio at least greater than 1·4 or less than 0·66 were deemed potentially significant and selected for further study. Unless otherwise stated, proteins were identified by their Uniprot database accession number (http://www.uniprot.org/).

RT-PCR and qRT-PCR expression profiles of candidate genes

Grapevine predicted genomic sequences (Jaillon *et al.*, 2007), revealing 95–100% homology to the microarray 70-mer oligonucleotides, were used to design gene-specific primers located in the 3′-UTR region and in the penultimate exon with PRIMER 3 and NETPRIMER softwares. These primers were then synthesized by Operon. Primer sequences and predicted product size are given in Table 1.

Semiquantitative RT-PCR reactions were performed following the protocol described in Camps *et al.* (2010). Quantitative RT-PCR (qRT-PCR) reactions were conducted in 25 μL final volume. The reaction mix contained 2 μL diluted RT product, 12·5 μL SuperMix iQ SYBR Green (Bio-Rad) and 0·2 μM each gene-specific primers, according to the manufacturer's instructions. Real-time PCR was performed on an iQ iCycler (Bio-Rad). Temperature cycling was as follows: 90 s at 95°C (initial denaturation step); 30 s at 95°C, 60 s at 60°C (40 amplification cycles) and a final melting curve from 60 to 95°C with a 0·5°C per 10 s slope). Data acquisition and analysis were done using the ICYCLER IQ software (v. 3.0a, Bio-Rad). Elongation factor 1 isoform γ (EF1γ, GenBank AF176496), actin (GenBank AY847627) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, GenBank CB973647) were used as housekeeping genes to calculate transcript relative gene expression using the GENEX (Gene Expression Analysis for iCycler iQ real-time PCR detection system) EXCEL applet from Bio-Rad. Each reaction was performed in triplicate on two independent RT products, and the statistical significance of the results was assessed using ANOVA analysis followed by a Student–Newman–Keuls post hoc test at the 0·05 threshold.

Results

Identification of differentially expressed genes between infected plants without symptoms and symptomless plants with negative pathogen recovery (S^-R^+/S^-R^-)

The comparison between infected plants without symptoms (S^-R^+) and symptomless plants with negative pathogen recovery (S^-R^-) was conducted under greenhouse and vineyard conditions. The purpose of this analysis was to attempt to identify genes that may be useful for developing an early and non-destructive diagnostic tool.

With a threshold of 1·4 for up-regulation and 0·66 for down-regulation and a P-value lower than 0·05, the number of up-regulated or down-regulated genes in S^-R^+ plants compared to S^-R^- plants were 102 and 77 respectively under greenhouse conditions and 131 and 169 under vineyard conditions (Fig. 2). Venn diagrams were constructed to identify genes that exhibited the same behaviour in greenhouse and vineyard conditions. Twenty-three genes differentially expressed for the comparison S^-R^+/S^-R^- were common to both greenhouse and vineyard conditions. Six of them were up-regulated and 17 were down-regulated in S^-R^+ greenhouse and vineyard plants compared with the corresponding symptomless plants with negative pathogen recovery (S^-R^-) plants. Among these genes, 17 (five up-regulated and 12 down-regulated) could be identified by mapping the corresponding oligonucleotide probes on the Pinot noir (PN 40024) grapevine genome (Jaillon *et al.*, 2007) and showed a good homology with known genes (Table 2).

Two genes up-regulated in S^-R^+ plants are involved in energy production. The *Vv10s0116g00060* locus encodes a protein involved in the chloroplast electron transport chain: a NADPH-quinone oxidoreductase subunit H. The *Vv13s0064g00900* gene has good homology with the *Arabidopsis* gene *At5g19855* that encodes for a protein RbcX. Three other genes *Vv18s0041g01220*, *Vv05s0094g00330* and *Vv02s0025g02640*, also up-regulated in S^-R^+ plants, are associated with *At1g72030, VvU97521* and *At1g64680* genes, respectively, and encode an acyl-CoA N-acyl transferase (GNAT) of GCN5 type, a class IV endochitinase and a protein with unknown function.

Several genes involved in carbon metabolism were down-regulated in the greenhouse and vineyard S^-R^+ plants. Thus, *Vv08s0007g03430* has a high homology

Table 1 Sequences and melting temperatures of primers used for RT-PCR (a) or qRT-PCR (b) of candidate genes selected after microarray analysis. (c) Sequences and melting temperatures of primers used for reference genes in RT-PCR and qRT-PCR

(a) Primers used for RT-PCR reactions

Grapevine transcripts

Probe ID	G12X ID	TC	Sense primer (5′–3′)	Tm (°C)	Antisense primer (5′–3′)	Tm (°C)	Product size (bp)
Vv_10003056	*Vv18s0089g01230*	TC107350	AGCTGCTTGTGGTCACTGATG	58·36	TGAATTGGTTGGGAGTGCTG	58·99	266
Vv_10004211	*Vv06s0004g04860*	TC120342	ACGGGAGGAAAAGGAGACAG	58·18	TGGCATGGTGGGGTATCTAA	58·52	471
Vv_10010533	*Vv01s0150g00460*	TC109065	CAGCCCCTCCATTCTTATCA	57·52	CCTACCAAAACCTCCCCAAT	58·14	675
Vv_10010940	*Vv10s0116g00060*	TC127687	ATCGGACCATCCATAGCAGT	56·66	ATTCCACCCCCAAAAACC	56·68	324
Vv_10012092	*Vv13s0064g00900*	TC115375	CTGTTGGAGGATGGTTGGAG	57·43	TATTTGCTGCGACGAAGTTG	57·51	461
Vv_10008923	*Vv02s0025g02640*	TC105859	GTGGGTGTGTGGGAATGTG	56·53	CTGACCGAGGAACTACGAGAA	57·04	407

(b) Primers used for qRT-PCR reactions

Grapevine transcripts

Probe ID	G12X ID	TC	Sense primer	Tm (°C)	Antisense primer	Tm (°C)	Product size (bp)
Vv_10003056	*Vv18s0089g01230*	TC107350	AGGATGAGGGGAAACTGAAG	60·4	TGAATTGGTTGGGAGTGCTG	60·4	101
Vv_10004211	*Vv06s0004g04860*	TC120342	GGGAATTTCAAGATCTCGACT	58·66	TGGCATGGTGGGGTATCTAA	60·4	110
Vv_10010533	*Vv01s0150g00460*	TC109065	ATCGCTGTCGTTCAGAGTCA	60·4	ACCCTGAAGTTTTTGCCGCT	60·4	111
Vv_10010940	*Vv10s0116g00060*	TC127687	TCTTTGGACCGTTGGAGATG	60·4	GTACCATGACGTATCTAGAATA	57·08	91
Vv_10012092	*Vv13s0064g00900*	TC115375	CTACTTCACATTCAAGGCTGT	58·66	GCTTATTTGCTGCGACGAAG	60·4	101
Vv_10008923	*Vv02s0025g02640*	TC105859	CAGGTCTCTGAGATAAACAATT	57·08	GGGCAAAATTTCAGTATCCTG	58·66	124

(c) Primers used as reference

Control transcripts

GenBank accession	Name	Sense primer	Tm (°C)	Antisense primer	Tm (°C)	Product size (bp)
AF176496	EF1g	CAAGAGAAACCATCCCTAGCTG	60	TCAATCTGTCTAGGAAAGGAAG	60	92
AY847627	Actin	CTTGCATCCCTCAGCACCTT	60	TCCTGTGGACAATGGATGGA	60	82
CB973647	GAPDH	CCACAGACTTCATCGGTGACA	60	TTCTCGTTGAGGGCTATTCCA	60	70

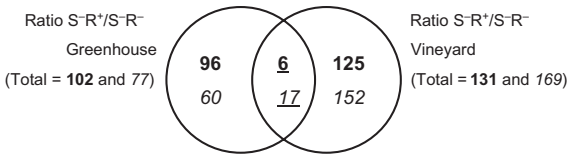

Figure 2 Venn diagram showing the distribution of genes differentially expressed (*P*-value ≤0·05 and threshold ≥1·4) between infected plants without symptoms (S^-R^+) and apparently uninfected plants (S^-R^-) grown in the greenhouse and in the vineyard. The numbers of up- and down-regulated genes in infected plants without symptoms (S^-R^+) compared to apparently uninfected plants (S^-R^-) are indicated in bold and italics respectively. The number of differentially expressed genes that are found in common between greenhouse and vineyard conditions is underlined at the intersection of the corresponding circles. Total numbers refer to the sum of up- and down-regulated genes for a given growth condition.

with an *A. thaliana* gene (*At3g10080*) that encodes a germin-like protein (Q9SR72). *Vv18s0089g01230* and *Vv11s0016g00470* are associated with a fructokinase 2 (O82616) and a sucrose synthase (P13708), respectively. These two enzymes are involved in the sucrose/starch balance and sucrose degradation pathway. Several genes involved in cell wall biosynthesis were also down-regulated in the greenhouse and vineyard S^-R^+ plants. The *Vv06s0004g03050* is homologous to a gene that encodes an arabinogalactan protein from *Gossypium hirsutum* (A9XTK6) and the *VvGRP68* gene (*Vv00s0187g00160*) encodes a cell wall structural protein. *Vv06s0004g04860* and *Vv01s0150g00460* are associated with genes encoding enzymes implicated in cell wall softening: an expansin (Q48818) and a xyloglucan endotransglucosylase (XET, Q38696). *Vv02s0154g00300*, *Vv04s0008g03930*, *Vv14s0108g00810* and *Vv03s0063g02360* were also down-regulated in infected plants without symptoms compared to symptomless plants with negative pathogen recovery. *Vv02s0154g00300* is homologous to the tobacco *NtEIG-C29* gene, *Vv04s0008g03930* a RD22-like protein. *Vv14s0108g00810* encodes a mini zinc finger transcription factor and *Vv03s0063g02360* a ripening-induced protein (Q6VEQ6).

These genes, differentially expressed in the comparison S^-R^+/S^-R^-, are potentially useful for developing an early non-destructive *E. lata* diagnostic tool. They are associ-

Table 2 Functional classification of the genes differentially expressed (ratio ≥ 1·54 and *P*-value ≤0·05) between S^-R^+ and S^-R^- plants, in greenhouse (G) and vineyard (V) conditions

				Expression profile		Greenhouse (G) S^-R^+/S^-R^-		Vineyard (V) S^-R^+/S^-R^-	
Probe ID	G12X ID	Protein ID	Annotation	Regulation	Condition	Ratio	*P*-value	Ratio	*P*-value
Vv_10010940	*Vv10s0116g00060*	Q0ZIW3	[VITVI] NADPH-quinone oxidoreductase subunit 1 chloroplast complete	UP	G + V	1·402	0·0004	1·489	0·0126
Vv_10012092	*Vv13s0064g00900*	B4VHV7	[ARATH] RbcX protein partial (63%)	UP	G + V	1·431	0·0002	1·411	0·0139
Vv_10013427	*Vv18s0041g01220*	Q9C7G6	[ARATH] GCN5-related acyl-CoA N-acyltransferase (GNAT) family, partial (43%)	UP	G + V	1·580	5·35E-05	1·415	0·0172
Vv_10000136	*Vv05s0094g00330*	O24530	[VITVI] Class IV endochitinase, complete	UP	G + V	1·740	0·0003	1·539	0·0061
Vv_10008923	*Vv02s0025g02640*	Q9SGU7	[ARATH] At1g64680 complete	UP	G + V	1·551	0·0001	1·418	0·0187
Vv_10004763	*Vv08s0007g03430*	Q9SR72	[ARATH] Germin-like protein partial (88%)	DOWN	G + V	0·685	0·0038	0·430	0·0007
Vv_10003056	*Vv18s0089g01230*	O82616	[ARATH] Fructokinase-5 complete	DOWN	G + V	0·709	0·0010	0·439	0·0005
Vv_10000177	*Vv11s0016g00470*	P13708	[GLYMA] Sucrose synthase, partial (84%)	DOWN	G + V	0·688	0·0058	0·543	0·0018
Vv_10000173	*Vv00s0187g00160*	Q9M4H6	[VITVI] Ripening-related protein, complete	DOWN	G + V	0·613	0·0197	0·627	0·0049
Vv_10004211	*Vv01s0150g00460*	Q38696	[ACTDE] Xyloglucan endotransglycosylase, complete	DOWN	G + V	0·549	7·37E-05	0·554	0·0023
Vv_10001696	*Vv06s0004g03050*	A9XTK6	[GOSHI] Fasciclin-like arabinogalactan protein 1, partial (72%)	DOWN	G + V	0·685	0·0004	0·447	0·0005
Vv_10010533	*Vv06s0004g04860*	Q48818	[ARATH] Expansin partial (91%)	DOWN	G + V	0·618	9·81E-05	0·580	0·0048
Vv_10011060	*Vv02s0154g00300*	Q9FXS2	[NICTA] NtEIG-C29, complete	DOWN	G + V	0·688	0·0003	0·652	0·0127
Vv_10011061	*Vv02s0154g00300*	Q9FXS2	[NICTA] NtEIG-C29, complete	DOWN	G + V	0·677	0·0009	0·624	0·0082
Vv_10003937	*Vv04s0008g03930*	Q4VT47	[VITVI] RD22, partial (69%)	DOWN	G + V	0·626	0·0015	0·610	0·0319
Vv_10010706	*Vv14s0108g00810*	A0ZXL1	[ARATH] Mini zinc finger protein, partial (84%)	DOWN	G + V	0·648	0·0003	0·602	0·0031
Vv_10004611	*Vv03s0063g02360*	Q6VEQ6	[VITVI] Ripening-induced protein 1 precursor partial (73%)	DOWN	G + V	0·690	0·0002	0·618	0·0051

ated with either response to infection (because infected plants R^+ and uninfected plants R^- were compared) or symptom absence (because apparently uninfected plants were compared with infected plants without symptoms), but are not distinguished in this analysis.

Identification of markers more specifically associated with presence of symptoms, absence of symptoms or response to infection

In order to identify genes that can be used as robust diagnostic markers specifically associated with symptom absence or response to infection, the microarray data obtained for both greenhouse and vineyard conditions and all the following comparisons (S^+R^+/S^-R^-, S^-R^+/S^-R^-, S^+R^+/S^-R^+) were examined. For each comparison, genes considered in this section were found to be differentially expressed in both greenhouse and vineyard conditions. The comparison S^+R^+/S^-R^- revealed genes that are associated with presence of symptoms or response to infection (Fig. 3a). The comparison S^-R^+/S^-R^- highlighted genes associated with either absence of symptoms or response to infection (Fig. 3b). Because both types of plant are infected by *E. lata*, the comparison S^-R^+/S^+R^+ identified genes associated with either symptom externalization or symptom absence (Fig. 3c). Robust diagnostic genes specifically associated with response to infection are found in common between comparisons of S^+R^+/S^-R^- and S^-R^+/S^-R^- plants (Fig. 3d). Strong diagnostic genes specifically associated with symptom absence were shared between comparisons of S^-R^+/S^+R^+ and S^-R^+/S^-R^- plants (Fig. 3e). Strong marker genes specifically associated with symptom presence were shared between comparisons of S^-R^+/S^+R^+ and S^+R^+/S^-R^- plants (Fig. 3f). For each group of genes (symptom presence, response to infection, symptom absence) expression ratios obtained for the three com-

parisons (S^+R^+/S^-R^-, S^-R^+/S^+R^+, S^-R^+/S^-R^-) were visualized in Fig. 4 and allowed us to establish an expected expression profile between the different kinds of plants: S^-R^+, S^+R^+ and S^-R^-.

Genes specifically associated with symptom presence included four down-regulated and 65 up-regulated genes in infected plants with symptoms, S^+R^+ (Fig. 4a). Seven genes specifically involved in the response to infection were less expressed in both greenhouse and vineyard infected plants (S^+R^+ and S^-R^+) compared to symptomless plants with negative pathogen recovery (S^-R^-) (Fig. 4b). These genes include: *Vv01s0150g00460* (XET), *Vv08s0007g03430* (germin-like), *Vv06s0004g03050* (AGP), *Vv18s0089g01230* (fructokinase), *Vv06s0004g04860* (expansin), and two other sequences (*Vv8s0007g07980*, *Vv10s0003g03110*) with poor homology to non-*Vitis* database sequences. Three genes associated with symptom absence were more highly expressed in greenhouse and vineyard infected plants without symptoms (S^-R^+) compared to S^+R^+ and S^-R^- plants (Fig. 4c). They corresponded to *Vv10s0116g00060* (NADPH-quinone oxidoreductase subunit H), *Vv13s0064g00900* (RbcX protein), and *Vv02s0025g02640* (a predicted protein of unknown function), also identified as a potential diagnostic candidate. Finally, the *Vv02s0154g00300* (NtEIG-C29), and *Vv05s0094g00330* (endochitinase) were differentially expressed in greenhouse and vineyard in the three comparisons and showed a differential expression pattern between the three kinds of plants S^+R^+, S^-R^- and S^-R^+ (Fig. 4d).

Validation of expression profile of candidate genes by RT-PCR

Six candidate genes were selected and their expression profile was studied by RT-PCR. Three genes (*Vv18s0089g01230*, *Vv06s0004g04860*, *Vv01s0150g00460*) were more specifically associated with response to *E. lata* infection (Fig. 5a) and the other three (*Vv10s0116g00060*, *Vv13s0064g00900*, *Vv02s0025g02640*) with symptom absence (Fig. 5b).

The RT-PCR expression profile obtained for *Vv18s0089g01230* (fructokinase), *Vv06s0004g04860* (expansin), and *Vv01s0150g00460* (XET) (Fig. 5Aa) in response to *E. lata* infection confirmed their microarray profile (Fig. 4b). These genes had a lower expression in infected plants (S^+R^+ and S^-R^+) than in symptomless plants with negative pathogen recovery (S^-R^-) in both vineyard and greenhouse conditions. The RT-PCR expression profile obtained for *Vv10s0116g00060* (NADPH-quinone oxidoreductase subunit H), *Vv13s0064g00900* (RbcX protein) and *Vv02s0025g02640* (Fig. 5Ab) is also consistent with their microarray profile (Fig. 4c). These genes were more expressed in infected plants without symptoms (S^-R^+) than in infected plants with symptoms (S^+R^+) or apparently uninfected plants (S^-R^-).

The expression profile of these six candidate genes was also studied during infection of plants with other grapevine fungi (*E. necator*, *P. viticola*, *B. cinerea*) (Fig. 5b). With the same number of amplification cycles, these genes had a stronger expression in response to *E. lata* than in response to the other pathogens tested. Some genes have no response (*Vv01s0150g00460*) or a non-differential expression in response to pathogens other than *E. lata*. For example, *Vv02s0025g02640* and *Vv18s0089g01230* genes presented the same expression pattern between apparently uninfected and infected leaves in response to *E. necator* and *B. cinerea*. The genes that also responded to other pathogens often had a reverse expression profile compared to *E. lata*. Thus, *Vv18s0089g01230* and *Vv06s0004g04860* were less strongly expressed in plants infected by *E. lata* but seem

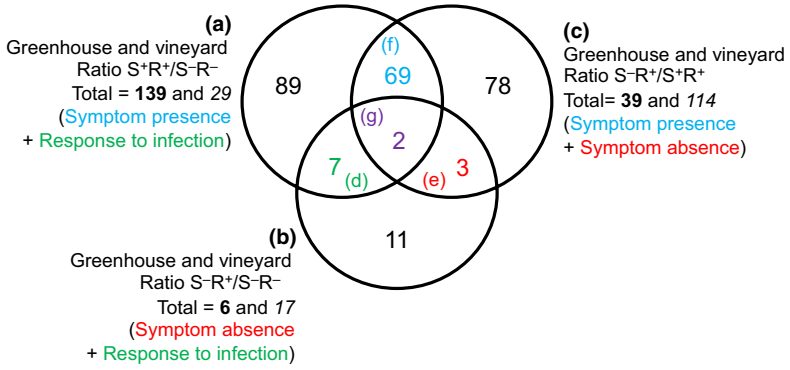

Figure 3 Venn diagram showing the distribution of genes differentially expressed (*P*-value ≤0·05 and threshold ≥1·4) between several conditions (greenhouse and vineyard) and several comparisons: (a) S^+R^+/S^-R^- (infected plants with symptoms and apparently uninfected plants), (b) S^-R^+/S^-R^- (infected plants without symptoms and apparently uninfected plants), and (c) S^-R^+/S^+R^+ (infected without symptoms and infected plants with symptoms). Total numbers refer to up- (bold) and down-regulated (italic) genes for a given comparison. (d) Genes more specifically associated with response to infection are common between the comparisons (S^+R^+/S^-R^-) and (S^-R^+/S^-R^-). (e) Genes more specifically associated with symptom absence are common between comparisons (S^-R^+/S^+R^+) and (S^-R^+/S^-R^-). (f) Genes more specifically associated with symptom presence are common between comparisons (S^+R^+/S^-R^-) and (S^-R^+/S^+R^+). (g) Genes common between the three comparisons. The number of differentially expressed genes that are found in common between different comparisons is given at the intersection of the corresponding circles.

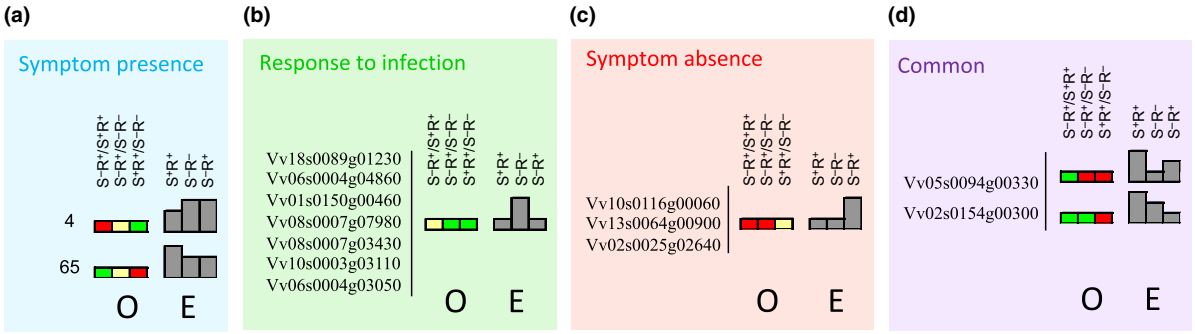

Figure 4 Identification of the genes associated with symptom presence (a), response to infection (b), symptom absence (c), and common between all comparisons (d). O, expression ratios obtained for the three comparisons (S^+R^+/S^-R^-, S^-R^+/S^+R^+, S^-R^+/S^-R^-) with red or green squares being associated respectively to an induction or repression pattern, and yellow squares indicating no differential expression of the gene; E, expected RT-PCR expression profiles between the different classes of plants: S^-R^+, S^+R^+ and S^-R^-.

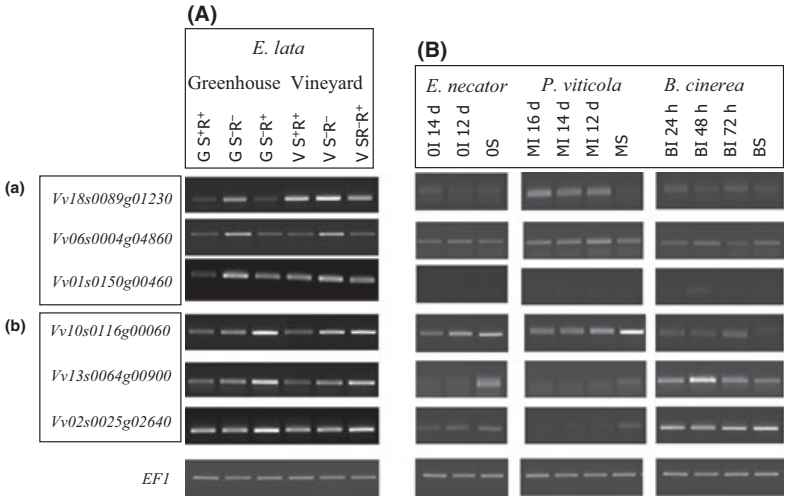

Figure 5 Semi quantitative RT-PCR expression profiles of six candidate genes potentially useful to develop an *Eutypa lata* diagnostic tool: (a) three genes more specifically associated with response to infection and (b) three genes more specifically associated with symptom absence. (A) Response to *E. lata*. The expression was studied with the same plants as those used for microarray analysis in greenhouse and vineyard conditions: infected grapevine showing symptoms (S^+R^+), infected grapevine showing no symptoms (S^-R^+), uninfected grapevine (S^-R^-). (B) Response to other pathogens: *Erysiphe necator*, *Plasmopara viticola*, *Botrytis cinerea*. OS, MS, BS are control non-inoculated plants. OI, plants collected 12 or 14 days after inoculation with *E. necator*. MI, plants collected 12, 14 or 16 days after inoculation by *P. viticola*. BI, plants collected 24, 48 or 72 h after inoculation by *B. cinerea*.

to be over-expressed in leaves infected with *E. necator* (*Vv06s0004g04860*, Fig. 5B OI 12d) or *P. viticola* (*Vv06s0004g04860* Fig. 5B MI 12d, *Vv18s0089g01230* Fig. 5B MI 12d 14d 16d). Similarly, *Vv10s0116g00060*, *Vv13s0064g00900* and *Vv02s0025g02640* were up-regulated in S^-R^+ grapevine in response to *E. lata* but were down-regulated after infection by *E. necator* (*Vv10s0116g00060* Fig. 5B OI 14d, *Vv13s0064g00900* Fig. 5B OI 12d 14d) or *P. viticola* (*Vv10s0116g00060*, *Vv13s0064g00900* and *Vv02s0025g02640* Fig. 5B MI 12d 14d 16d). Some genes (*Vv13s0064g00900* and *Vv06s0004g04860*) had similar expression profiles for *E. lata* and *B. cinerea*, but this response was limited to one time of the infection kinetic (Fig. 5B, BI 48 h and BI 72 h, respectively).

Validation of expression profile of candidate genes by qRT-PCR

The expression of the same six candidate genes was also assessed by quantitative RT-PCR (qRT-PCR; Fig. 6). The statistical significance of the results was assessed using a Kruskal–Wallis analysis followed by a Student–Newman–Keuls post hoc test at the 0·05 threshold. Except for *Vv10s0116g00060* in the greenhouse, which did not seem differentially expressed between the three kinds of plants characterized, the profile obtained by qRT-PCR for these genes was consistent with the profile obtained by RT-PCR.

Vv18s0089g01230 (fructokinase), *Vv06s0004g04860* (expansin) and *Vv01s0150g00460* (xyloglucan endo-

transglycosylase 2) were significantly down-regulated in infected plants with or without symptoms (S^+R^+ and S^-R^+) compared to symptomless plants with negative pathogen recovery (S^-R^-) in both greenhouse and vineyard conditions. This is the expected profile for infection markers. For these genes, infected plants with or without symptoms (S^+R^+ and S^-R^+) were associated in the same group (a) while apparently uninfected grapevine (S^-R^-) belonged to another statistically different group (b). *Vv02s0025g02640* and *Vv13s0064g00900* (RbcX) were significantly up-regulated in infected symptomless grapevines (S^-R^+) compared to symptomless plants with negative pathogen recovery (S^-R^-) and infected plants with symptoms (S^+R^+) in both greenhouse and vineyard conditions. *Vv10s0116g00060* (NADPH-quinone oxidoreductase subunit H) was significantly up-regulated in infected symptomless grapevine (S^-R^+) compared to symptomless plants with negative pathogen recovery (S^-R^-) and infected symptomatic plants (S^+R^+) in vineyards. This is the expected profile for markers associated with symptom absence. For these genes, infected plants with S^+R^+ and apparently uninfected grapevine (S^-R^-) were associated in the same group (a) while symptomless infected plants (S^-R^+) were in another statistically different group (b).

Discussion

Eutypa dieback diagnosis is generally based on the *in planta* detection of the fungus (Rolshausen *et al.*, 2004). Diagnosis can also be achieved from compounds secreted by the fungus (Octave *et al.*, 2009), but the most common method of diagnosis is based on observation of foliar symptoms and quantification. This technique is only informative after several years of survey because there are considerable interannual variations in symptom expression. It is not always possible to identify *E. lata* infection through observation of characteristic brown sectorial necrosis on longitudinal sections of the grapevine trunk. Sectorial necroses associated with Botryosphaeriaceae species may look the same as those caused by *E. lata* and can only be differentiated after the fungus is isolated in pure culture. Furthermore, *in vitro*, some Diatrypaceae species are indistinguishable from their morphological characters (Trouillas *et al.*, 2010) and can only be identified with certainty by molecular techniques.

In the past few years, different DNA-based markers have been developed to identify *E. lata* via direct PCR on DNA extracted from grapevine necrotic wood (Lecomte *et al.*, 2000; Lardner *et al.*, 2005), or indirect PCR on DNA extracted from mycelium growing from a piece of necrotic wood cultured *in vitro* (Lecomte *et al.*, 2000; Rolshausen *et al.*, 2004; Lardner *et al.*, 2005). Although PCR detection of *E. lata* may provide a robust diagnostic test, this is a destructive assay requiring the use of perennial grapevine trunk tissues. Besides, this method relies on the design of specific primers for *E. lata*, and problems of primer specificity have been largely discussed in the literature cited above.

Alternatively, HPLC identification of secondary metabolites secreted by *E. lata* has been suggested for diagnosis (Mahoney *et al.*, 2005; Lardner *et al.*, 2006; Rolshausen *et al.*, 2008). However, working *in vivo*, metabolites of *E. lata* have not been detected so far in various plant organs (inflorescences, leaves, berries; Mahoney *et al.*, 2005) nor in the sap (Lardner *et al.*, 2006) of infected grapevines with or without symptoms. Besides, these HPLC detection methods need to consider potential false positive identification, because other fungi involved in wood decay diseases might also secrete components structurally related to those synthesized by *E. lata*.

A third approach, based on serology, has also been used to identify *E. lata*. Antibodies have been raised against *E. lata* ascospores and hyphal material and used to identify the fungus by immunodiffusion and immunofluorescence (Francki & Carter, 1970). More recently, it has been shown that *E. lata* secretes various polypeptides (Octave *et al.*, 2006a,b) and enzymes (Schmidt *et al.*, 1999) into its culture medium. Rabbit antibodies raised against these polypeptides were used to develop a sensitive and specific serological assay for the *in planta* identification of *E. lata* (Octave *et al.*, 2009). However, a major drawback with this method is that the antibody supply is limited, new pools of antibodies have to be prepared on a regular basis, requiring repeated host selection and drastic quality control (Octave *et al.*, 2009).

The present work is, to the best of the authors' knowledge, the first one in which a different detection approach has been taken, based on the identification of grapevine genes differentially expressed in response to *E. lata*.

The first comparison focused on was S^-R^+/S^-R^-, between infected plants without symptoms (S^-R^+) and symptomless plants with negative pathogen recovery (S^-R^-) that had been grown in greenhouse or vineyard conditions. The purpose of this analysis was to identify genes potentially useful to develop an early and non-destructive diagnostic tool. The low number of genes differentially expressed in this comparison and common between greenhouse and vineyard conditions (six up- and 17 down-regulated genes, respectively) is not surprising. Microarrays were conducted with leaf material (distant from the initial infection location), collected from infected plants without symptoms and from apparently uninfected plants (symptomless plants with negative pathogen recovery).

Among the genes that were over-expressed in S^-R^+ plants, the loci *Vv13s0064g00900, Vv18s0041g01220* and *Vv05s0094g00330* were identified. *Vv13s0064g00900* has a good homology with the *Arabidopsis* gene *At5g19855* that encodes for a RbcX protein. The active RuBisCO is a protein complex of eight large subunits $(RbcL)_8$ associated with eight small subunits $(RbcS)_8$. The RbcX protein allows the formation of $(RbcL)_8$ complex which is afterwards spontaneously associated with the small subunits to constitute active RuBisCO (Saschenbrecker *et al.*, 2007). *Vv18s0041g01220* has a good homology with the *Arabidopsis* gene *At1g72030* that encodes an acyl-CoA N-acyl transferase (GNAT) of

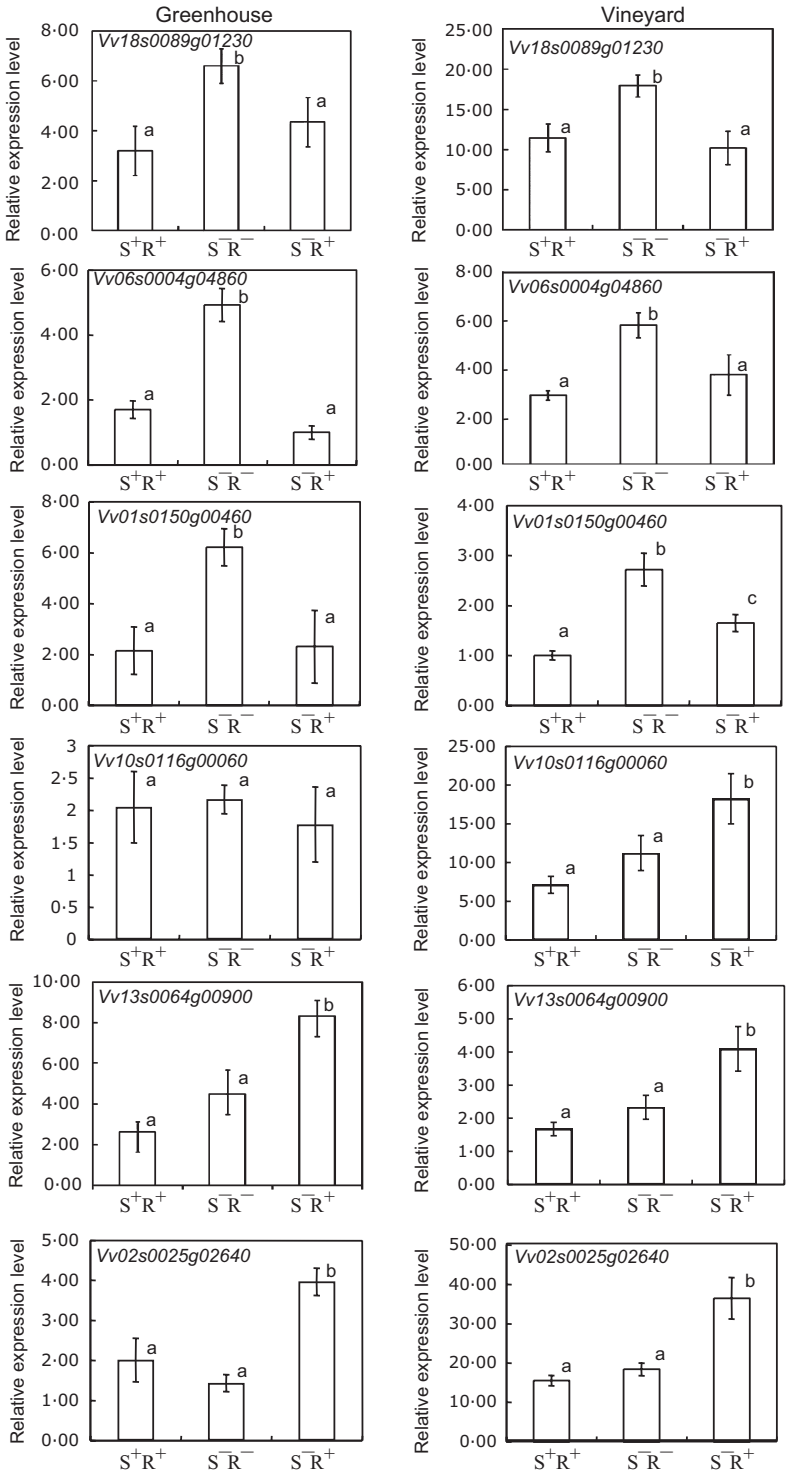

Figure 6 qRT-PCR expression profile of six candidate genes potentially useful to develop a tool for diagnosis of *Eutypa lata*. Three genes are more specifically associated with response to infection and three genes are more specifically associated with symptom absence. The small letters on the bar diagrams represent the results of a Student–Newman–Keuls test at the 0·05 threshold. The *y*-axis gives the relative expression level of the candidate gene (changes in steady-state mRNA levels of this gene across multiple samples relatively expressed to the levels of internal control RNA of housekeeping genes).

GCN5 type. GCN5 proteins are acetyl transferase histones that regulate histone acetylation and thus DNA accessibility. Members of the GNAT family are implicated in the regulation of cell growth and development. Their importance in these processes is probably related to their role in transcription and DNA repair. *Vv05s0094g00330* has a good homology with the *Vitis vinifera* gene *VvU97521* that encodes a class IV endochitinase. *VvU97521* expression is also increased during grape berry ripening (Robinson *et al.*, 1997), or in transgenic grapevines overexpressing the transcription factor *VvWRKY1* (Marchive *et al.*, 2007). Moreover, these transgenic plants are less susceptible to infection by *P. viticola* (Marchive *et al.*, 2007).

Among the genes that were under-expressed in S^-R^+ plants, the loci *Vv18s0089g01230*, *Vv11s0016g00470*, *Vv00s0187g00160* and *Vv02s0154g00300* were identified. *Vv18s0089g01230* and *Vv11s0016g00470* are associated with a fructokinase 2 (O82616) and a sucrose synthase (P13708), respectively. These two enzymes are involved in the sucrose/starch balance and sucrose degradation pathway. Down-regulation of *Vv18s0089g01230* and *Vv11s0016g00470* in S^-R^+ plants should promote the degradation of starch to the benefit of sucrose. The *VvGRP68* gene (*Vv00s0187g00160*) encodes for a cell wall structural protein, the expression of which changes during grape berry maturation (Davies & Robinson, 2000). *Vv02s0154g00300* is homologous to the tobacco *NtEIG-C29* gene, isolated by SSH (suppression subtractive hybridization) from tobacco leaves treated with an elicitor from the oomycete *Phytophthora infestans* (Takemoto *et al.*, 2003). It encodes a small hydrophobic protein (Q9FXS2) with typical HPS (soybean hydrophobic protein) domains. The HPS family includes alpha-amylase inhibitors and LTP (lipid transfer proteins) (Weyman *et al.*, 2006).

The genes differentially expressed in the comparison S^-R^+/S^-R^- can be associated with either response to infection (because infected plants were compared with uninfected plants) or symptom absence (because apparently uninfected plants were compared with infected plants without symptoms), but they are not distinguished in this specific analysis. Both these categories of genes can be potentially useful to develop an early and non-destructive diagnostic tool. They could be used for early diagnosis because the transcriptomic modifications induced by *E. lata* were detected in the sensitive cultivar Cabernet Sauvignon that did not yet show leaf symptoms. This diagnosis would be non-destructive because it uses leaf material rather than wood (trunk) samples.

In order to identify strong diagnostic markers specifically associated with symptom absence or response to infection, the microarray data sets obtained for both greenhouse and vineyard conditions and all comparisons (S^+R^+/S^-R^-, S^-R^+/S^-R^-, S^+R^+/S^-R^+) were combined. Seven strong diagnostic genes specifically associated with response to infection and three strong diagnostic genes specifically associated with symptom absence were identified. These genes are strong markers because they were differentially expressed in several conditions (both greenhouse and vineyard) and also several comparisons. They can be more specifically associated with response to infection because they were common between the comparisons (S^+R^+/S^-R^-) and (S^-R^+/S^-R^-). In addition, genes more specifically associated with symptom absence were found to be common between comparisons (S^-R^+/S^+R^+) and (S^-R^+/S^-R^-).

The RT-PCR expression profile for six of these candidate genes, obtained in response to *E. lata* infection, mostly confirmed their microarray profile. Although these genes also respond to other grapevine pathogens (*E. necator*, *P. viticola*, *B. cinerea*), they often have an opposite transcription response when compared to their response to *E. lata*, and if the response is the same it is limited to a specific time of the infection kinetic (i.e. not consistent throughout the infection). Therefore, the response to *E. lata* could be distinguished from possible responses to these other pathogens. Based on RT-PCR (*E. lata* and other fungal pathogens) and qRT-PCR (*E. lata*) expression analysis, grapevine genes *Vv18s0089g01230*, *Vv06s0004g04860*, *Vv01s0150g00460*, *Vv13s0064g00900*, *Vv02s0025g02640* transcript levels could prove to be useful to develop an early and non-destructive *E. lata* diagnostic tool that could be used in both greenhouse and vineyard conditions, with applications for field, research, and nursery purposes. The sensitivity, reproducibility, cost and time of diagnostic tools directly (PCR tests) or indirectly (serology kits) based on these findings still need to be investigated.

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