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ORIGINAL PAPER

Gene expression associated with compatible viral diseases in grapevine cultivars

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Abstract Viral diseases affect grapevine cultures without inducing any resistance response. Thus, these plants develop systemic diseases and are chronically infected. Molecular events associated with viral compatible infections responsible for disease establishment and symptoms development are poorly understood. In this study, we surveyed viral infection in grapevines at a transcriptional level. Gene expression in the *Vitis vinifera* red wine cultivars Carménère and Cabernet-Sauvignon naturally infected with GLRaV-3 were evaluated using a genome-wide expression profiling with the *Vitis vinifera* GeneChip® from Affymetrix. We describe numerous genes that are induced or repressed in viral infected grapevines leaves. Changes in gene expression involved a wide spectrum of biological functions, including processes of translation and protein targeting, metabolism, transport, and cell defense. Considering cellular localization, the membrane and endomembrane systems appeared with the highest number of induced genes, while chloroplastic genes were mostly repressed. As most induced genes associated with the membranous system are involved in transport, the possible effect of virus in this process is discussed. Responses of both cultivars are analyzed and the results are compared with published data from other species. This is the first study of global gene profiling in grapevine in response to viral infections using DNA microarray.

Keywords Vitis · GLRaV-3 · Affymetrix · Microarrays · Gene profiling

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Introduction

Grapevine is an ancient culture that currently constitutes the most economically important fruit species worldwide. Diverse pathogens attack this species causing physiological disorders that finally lead to yield losses. Among them, viral diseases can have a serious impact on grapevine productivity and fruit quality. Viral infections are usually disseminated through biological vectors and vegetative propagation of virus-infected material. These infections affect the vegetative organs inducing foliar deformations, alterations in leaf color, and, in some cases, graft rejection (Martelli 1993). Severe infections also reduce berry setting and cause irregular and delayed ripening (Martelli 1993). Among the more than 40 different viruses that infect grapevines (Martelli 1993), the leaf roll-associated closterovirus-3 (GLRaV-3) is one of the most wide spread. GLRaV-3 belongs to the *Ampelovirus* genus of Closteroviridae and is a positive-sense ssRNA virus transmitted by grafting and mealy bugs (Ling et al. 2004). Diseases

associated with this virus affect fruit quality by delaying ripening, diminishing sugar content, and, in red cultivars, reducing color (Borgo and Angelini 2002).

Grapevines develop an active resistance response against pathogens like fungi and bacteria, and some resistance genes have been identified (Aziz et al. 2003; Vivier and Pretorius 2002; Di Gaspero and Cipriani 2002). To date, no active resistance response against viral diseases has been found in grapes, and viruses establish a compatible interaction causing disease spread through all plant tissues. However, it has been shown that diverse susceptible hosts are not passive against a pathogen and can set up a defense response that could be less intense and not enough to stop viral replication and dissemination (O'Donnell et al. 2003; Ehrenfeld et al. 2005). Visible plant symptoms represent the sum of molecular, cellular, and physiological changes induced by viruses that are part of the host defense response. In compatible interactions, multiple changes in gene expression underlie the disease symptom development and also control the levels of virus in infected tissues (Maule et al. 2002).

The DNA microarray techniques that permit the simultaneous analysis of a large number of genes are now contributing to understanding the plant–pathogen relationship (Wan et al. 2002). Studies of compatible interactions in the model plant *Arabidopsis thaliana* have shown that different viruses induce plant gene expression changes on a wide array of cellular processes (Golem and Culver 2003; Whitham et al. 2003). These changes include the induction of genes associated with defense and stress, signal transduction, and metabolism (Whitham et al. 2003). On the other hand, a shut-off phenomenon in host gene expression has been observed during compatible interactions affecting different cellular process (Maule et al. 2002; Golem and Culver 2003, Whitham et al. 2003).

With the aim to investigate the effect of viral infections on the grapevine, transcript profiles of the red wine cultivars Carménère and Cabernet-Sauvignon naturally infected with GLRaV-3 were compared with virus-free grapevine plants. These two cultivars were chosen because both are susceptible to leaf roll disease, Cabernet-Sauvignon is one of the most economically valuable grapevine in the world, whereas Carménère has been acquiring relevance in Chile, which is reflected by the notable increase in the area cultivated during the past years. The application of genomic strategies to the functional study of grapevine is now possible due to the efforts in obtaining expressed sequenced tags and transcript analysis in these species (Goes da Silva et al. 2005). In the present study, we have used the recently developed *Vitis vinifera* GeneChip® microarray that contains 14,000 *V. vinifera* transcripts and 1,700 transcripts from other *Vitis* species. We describe in this paper the identification of numerous genes that are induced or repressed during viral infection in grapevine associated with two specific cultivars. The study contributes to understanding the changes triggered by viruses on grapevine physiology and to developing some proposals for mechanisms that infected grapevines utilize to contend the viral pathogen.

Materials and methods

Plant material

Healthy and GLRaV-3-infected grapevine plants were obtained from the nursery of Agronomy Faculty, P. Universidad Católica de Chile. Nine viruses that have a high incidence in Chile were screened by ELISA tests (Agritest) following manufacturer conditions: Grapevine Virus A (GVA), Grapevine virus B (GVB), Grapevine Fanleaf Virus (GFLV), Grapevine Fleck Virus (GFkV), Tomato Ringspot Virus (ToRSV), and Grapevine Leaf-Roll-Associated Viruses (GLRaV) 1, 2, 3, and 7. Healthy and virus-infected plants were maintained separately in greenhouses with controlled growing conditions. Medial leaves (approximately 8 cm diameter) from 12 plants were collected during growing season, to assure collecting leaves of about the same age. These included healthy Carménère and Cabernet-Sauvignon plants used as control and GLRaV-3 infected Carménère and Cabernet-Sauvignon plants used as infected tissue.

RNA extraction and RT-PCR

Total RNA was extracted from GLRaV-3-infected and healthy *Vitis vinifera* cv. Carménère and Cabernet-Sauvignon leaves following the Tris–LiCl method modified by Goes da Silva et al. (2005). Briefly, samples were extracted with homogenization buffer (200 mM Tris–HCl, pH 8.5, 1.5% (w/v) lithium dodecylsulfate, 300 mM LiCl, 10 mM sodium EDTA, 1% (w/v) sodium deoxycholate, 1% (v/v) Nonidet P-40, 2 mM aurintricarboxylic acid, 20 mM dithiothreitol, 10 mM thiourea and 2% (w/v) polyvinylpolypyrrolidone). After RNA precipitation with sodium acetate and isopropanol, samples were extracted with 25:24:1 phenol:chloroform:isoamyl alcohol and 24:1 chloroform:isoamyl alcohol before performing LiCl precipitations. Before RT-PCR reactions, samples were treated with DNase (Invitrogen). Then, first-strand cDNA was synthesized with random hexamers and reverse transcriptase (Stratagene). An aliquot of 1 μl was used as template for PCR. For GLRaV-3 confirmation, the primers C50 and H59 that amplify 945 bp of coat protein (Minafra and Hadidi 1994) were used.

Microarray hybridization

Six Carménère (three healthy and three infected) and six Cabernet-Sauvignon (three healthy and three infected) plants constituted the biological samples for expression analysis. RNA quality and concentration was measured using an Agilent 2100 bioanalyzer charging 2 μl of each sample in a RNA 600 Nano Lab Chip. Samples were processed following the GeneChip® Expression 3′-Amplification Reagents One-cycle cDNA synthesis kit instructions (Affymetrix Inc, Santa Clara, CA, USA). Single-stranded cDNA was synthesized with 6.5 μg of RNA of each sample, and oligo-dT-T_7Promoter Primers using the Superscript II (Invitrogen, USA). Then, double-stranded cDNA was synthesized and used as template to generate biotin-targeted cRNA following manufacturer's specifications. Fifteen micrograms of the biotin labeled cRNA was fragmented to strands between 35 and 200 bases in length. The fragmented cRNA (10 μg) was hybridized on a *Vitis vinifera* GeneChip® genome array (http://www.affymetrix.com/products/arrays/specific/vitis.affx) using standard procedures (45°C for 16 h). The chip is composed of approximately 14,000 *V. vinifera* probe sets and 1,700 probe sets of other *Vitis* species and hybrids, allowing the exploration of about 30–50% of the grape transcriptome. Chip probes were designed from public available databases that contain sequences from cDNA libraries of different *V. vinifera* cultivars, mainly Cabernet-Sauvignon and Chardonnay, among others. Libraries include sequences of several developmental stages and tissues, such as berry, leaf, flowers, and roots, and also contain ESTs from different conditions or treatments, comprising biotic or abiotic stresses. All sequences are accessible at Affymetrix web site (http://www.affymetrix.com/products/arrays/specific/vitis.affx) and in grapevine databases (http://www.barleybase.org and http://www.plexdb.org). Each transcript is represented by 16 pairs of 25-mer probes synthesized in situ on the array. The arrays were washed and then stained in a Fluidics Station 450. Scanning was carried out with the GeneChip® scanner 300 and image analysis was performed using GeneChip® Operating Software.

GeneChip® data processing and analysis

GeneChip® arrays data were first assessed using a set of standard quality control steps described in the Affymetrix manual *GeneChip® Expression Analysis: Data Analysis Fundamentals* (http://www.affymetrix.com/products/arrays/specific/vitis.affx). Calls of all three spike-in controls BioC, BioD, and Cre were present, and their intensity values increased from BioC to Cre, as expected. Average background values ranged from 29 to 106, and only two arrays had background values greater than 100, the suggested limit. The range of scaling factors was 1.1 to 5.9, with two arrays exhibiting scaling factors greater than the optional threshold of 5. Noise values, as measured by raw *Q*-values, varied between 0.8 and 3.1. Digestion curves displaying trends in RNA degradation between the 5′ end and the 3′ end in each probe set were also inspected, and all 12 proved very similar.

Arrays data were processed and normalized by RMA (Robust Multi-Array Average) (Irizarry et al. 2003) using the R package affy (Gautier et al. 2004). Specifically, expression values were computed from raw *CEL* files by first applying the RMA model of probe-specific correction of PM (perfect match) probes. These corrected probe values were normalized via quantile normalization, and a median polish was applied to compute one expression measure from all probe values. Resulting RMA expression values were log2-transformed. Both Pearson correlation and Spearman rank coefficients were computed on the RMA expression values (log2-transformed) for each set of biological replicates. Spearman coefficients ranged from 0.903 to 0.988 and Pearson coefficients ranged between 0.913 and 0.989.

To determine which genes were differentially expressed between each infected cultivar and its corresponding control state, an ANOVA was performed on the RMA expression values (Kerr et al. 2000). The following model was used for this analysis: $y_{ijk} = C_i + T_j + (CT)_{ij} + \varepsilon_{ijk}$, where y_{ijk} denotes the log2 signal measured for cultivar i, treatment j, and biological replicate k, with $1 \le i \le 2$, $1 \le j \le 2$, and $1 \le k \le 3$. The terms C_i and T_j measure the effect of the cultivar and treatment, respectively, and the interaction term $(CT)_{ij}$ accounts for the interaction between cultivar and treatment.

An ANOVA was performed on each gene using the linear model above and three contrasts based on differences between cultivars, and each cultivar's stress and control state. The R package limma was used for ANOVA methods (http://www.bioconductor.org/repository/devel/vignette/affy.pdf) A multiple-testing correction (Benjamini and Hochberg 1995) was applied to the p values of the F statistics to adjust the false discovery rate. Genes with adjusted F statistic p values <0.05 were extracted for further analysis.

Semiquantitative RT-PCR

For semi-quantitative RT-PCR (sqRT-PCR), 2.5 μg of total RNA DNase-treated were used to synthesize cDNA, as was previously described. PCR reactions were done with 1 μl of cDNA samples. A master mix was prepared for each gene and all sqRT-PCR were performed in biological triplicate, in an Eppendorf Mastercycler Gradient. To establish the kinetic of amplification of each gene, PCR reactions

covering from 19 to 44 cycles were performed. For all genes evaluated, the logarithmic amplification was observed with 31 cycles and this point was considered to compare gene expression. The following thermal cycle conditions were used: 94°C for 3 min, followed by 94°C for 50 s, 56°C for 50 s and 72°C for 3 min for 31 cycles. Amplified grapevine genes were lipid transfer protein (TC: 46011), harpin-induced 1 (TC: 40876), and tropinone reductase (TC: 40352).

Specific primers for the three genes (LTP-Vv-5′:CCA CACCATATGCAGAGGAG and LTP-Vv-3′:AATGG AGCTGAGCACATGTG; HIN1-Vv-5′:ATGCCATCACA GCAACACA and HIN1-Vv-3′:TCAGGGCCTCA TCTTCTCA; TROP-Vv-5′:GCTTCCTGCTCCAGATGC and TROP-Vv-3′:TGCCACCGTACACACCTG) were designed based on the sequences of the TC numbers (http://www.tigrblast.tigr.org/tgi/). All PCR products have an average length of 300 bp. Relative expression levels of each sample were normalized to the expression level of GPDH, TC: 44984 (primers GPDH-Vv-5′:CAGGATGC CATGTGGACAA and GPDH-Vv-3′:GTGTTGCC TTCATTGAATGG), which was expressed at a constant level in our experimental conditions. Quantification was done using Scion Image beta 4.0.2 software (Scion Corporation).

Results

Viral infection induces changes in grapevine transcript profiling in a wide spectrum of biological functions

Initially, virus-free and naturally GLRaV-3 infected grapevine plants were selected because the transmission of viral diseases to grapevine plants in controlled experimental conditions has turned out to be difficult to implement. The GLRaV-3 infection was confirmed by RT-PCR. The group of virus-free plants was consistently negative for all the grapevine viruses. Changes in gene expression in virus-infected grapevine plants were addressed in the cultivars Carménère and Cabernet-Sauvignon. In each case, three independent biological replicates were used and all hybridizations showed a similar distribution of expression values. After data normalization, medians distribution was uniform, reflecting that expression values were corrected for technical variances (Fig. 1). The percent of probe set that showed hybridization on the chips (percent call rate) ranged from 58 to 72% across all arrays, indicating that about 9,000–11,300 genes were expressed at detectable level in leaf tissues.

ANOVA showed 492 genes in the Carménère cultivar, and 1,216 genes in Cabernet-Sauvignon with significant differences between control and the infected state (Tables S1 and S2, respectively). Genes with absolute log-transformed differential expression values ≥1 were extracted for further inspection, resulting in 341 Carménère genes and 692 Cabernet-Sauvignon genes.

The assignment of biological roles permits an overview of the metabolic changes occurring during viral disease in grapevines (Fig. 2). This analysis was done considering the sequences of genes represented on the array and their annotation (Gene Ontology Consortium). The wide range of cellular processes affected by viral infection in both cultivars includes mainly functions related with protein turnover, transport, metabolism, transcription, cell defense, and an important number of genes belonging to the biological process unknown category.

Cellular changes in grapevine leaves infected with virus involved both induction and repression of gene expression (Fig. 3). In Carménère, 52 genes were induced and 289 were repressed in viral infected tissues. A more detailed analysis of these genes showed that some functional categories are exclusively represented by repressed genes, such as those involved in biosynthesis and cell cycle. In Cabernet-Sauvignon, 299 genes were induced and 393 were repressed in viral infected tissues. Overall, an important proportion of genes associated with unknown biological processes appear repressed. In contrast, other biological processes are predominantly induced, such as the genes involved in energy, transport, and signal transduction.

The most highly induced genes of virus-infected Carménère and Cabernet-Sauvignon plants are shown in Table 1 and Table 2, respectively. Among these genes, there is an important group that codes for stress- and defense-related proteins. The lipid transfer protein (LTP) that is specifically related to virus response is induced in both cultivars. Stress-responsive proteins like the patatin-like protein, the agenet domain containing protein and MAP kinase phosphatase (MKP1) are also induced. Aging genes like tropinone reductase and harpin-induced family protein (HIN-1), and the detoxifying gene gluthathione *S*-transferase (GST) were up-regulated. In petunia and maize, diverse GSTs are needed for efficient anthocyanin export from the site of synthesis in the cytoplasm into the vacuole for storage (Mueller et al. 2000). Over-expression of GST may correlate to the fact that GLRaV-3 infected leaves of grapevines usually turn red. Several developmental NAM family NAC2 proteins that are related to biotic and abiotic stress, among other genes related to sugar metabolism like sugar transporters and glycosyl transferase protein, are also induced. It is noteworthy that in Carménère cultivar there is

an important induction of sugar, oligopeptides, and hormone transporters (Table 1).

Gene induction is mostly associated with cell membrane and endomembrane system, while repressed genes are related to the chloroplast

Viral responsive host genes were classified according to cellular component. A relevant part of the induced genes turned out to be related with the membranous system, especially in the Carménère cultivar (Fig. 4). Genes related to the cell membrane (30.5%) and endomembrane system (23.5%) represent more than half of this group of induced genes. A more detailed analysis revealed that most of them code for proteins involved in the transport function. These include transporters of hormones (auxines and citoquinines), lipids, sugars, and oligopeptides. Also, this category contains genes of proteins associated with the remodeling of the cell wall like extensin and hidroxiproline-rich proteins that are anchored to the cell membrane.

On the other hand, 59% of the repressed genes are associated with the chloroplast in Carménère cultivar (Fig. 4). An important proportion of these genes codes for proteins associated with metabolic functions. These include photosynthetic proteins as well as photosystems constituents and chlorophyll biosynthesis enzymes. This group contains also ATP-dependent chloroplast protease and chaperones, such as the DNAJ domain-containing protein and the co-chaperone grpE. Some genes associated with the stress-oxidative-detoxification system are also part of this group.

Common response against infection with GLRaV-3 between cultivars

A comparison by means of ANOVA between gene changes in Carménère and Cabernet-Sauvignon plants that occur during viral infection with GLRaV-3 indicated that there were no significant differentially expressed genes. A global view of functions affected by the pathogen showed slight differences between cultivars. However, only 93 in common genes showed significant differences between healthy and virus-infected plants in both cultivars. This group represents the total of statistically significant genes that are responding to the infection without considering the change magnitude. Common genes that were induced and repressed, considering a twofold change cut-off, were functionally classi-

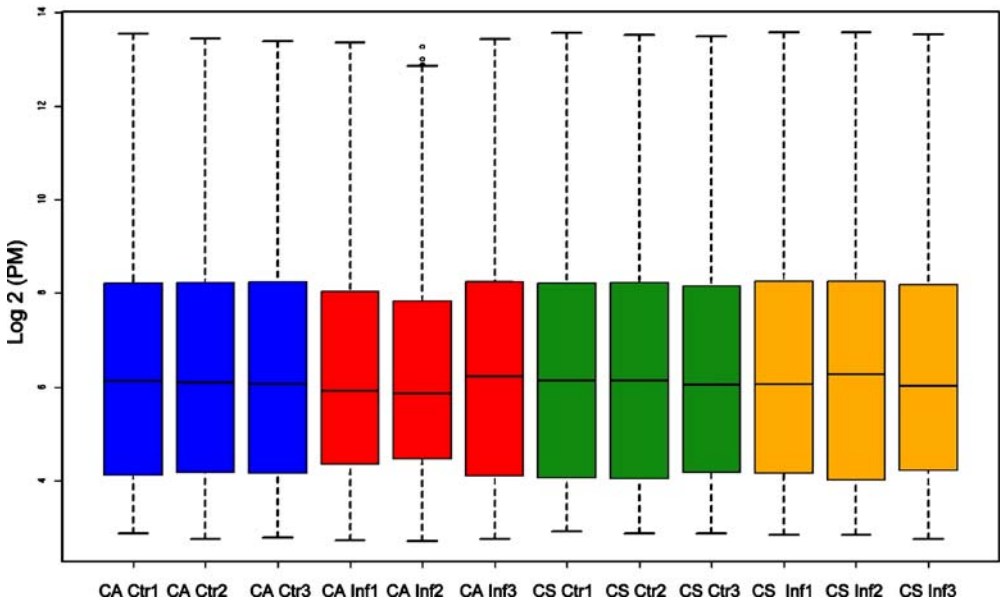

Fig. 1 Boxplots of normalized perfect match values. The figure shows data distribution of $\log_2$ perfect match (PM) values for all arrays after processing and normalization. Arrays were hybridized with the following samples: RNA from Carménère and Cabernet-Sauvignon control non-infected plants (CA-Ctrl and CS-Ctrl, respectively); RNA from Carménère and Cabernet-Sauvignon infected with GLRaV-3 (CA-Inf and CS-Inf, respectively). Numbers 1 to 3 represent biological independent replicates. Log_2 (PM) values between the 25th and the 75th percentiles are grouped into each *box*. *Upper and lower dotted lines* denote values greater and smaller than the 90th and the 10th percentiles

fied, and they are listed in Table 3. A great number of genes specifically related with photosynthesis were repressed in both cultivars

Verification of microarray data

The over-expression of genes belonging to the biological functions of cell rescue, defense, death, and aging was validated using semi-quantitative RT-PCR. Genes evaluated were: lipid transfer protein (LTP, TC46011), tropinone reductase (Trop, TC40352), and harpin-induced protein 1 (HIN1, TC40876). It was shown that GPDH behaves as a housekeeping gene in a broad repertory of biotic and abiotic conditions (Mahalingam et al. 2003). The selection of this gene as constitutive control was based on its expression level and the fact that no significant differences were found between virus-infected and control grapevine plants in our experimental conditions. To estimate the synthesized product, agarose gel images were quantified and the fold-change relative to a healthy control for each gene was calculated. The induction of LTP, Trop, and HIN 1 was confirmed in these plants by semiquantitative RT-PCR (Fig. 5).

Discussion

Transcript abundance of a broad spectrum of cellular functions is mostly repressed in compatible viral infection in grapevine

The study of a systemic viral infection in the cultivars Cabernet-Sauvignon and Carménère showed that a wide spectrum of grapevine biological functions was affected (Fig. 2). This reflects the fact that viruses are obligate intracellular parasites and that they must establish tight interactions with host cells, causing a general effect in cell physiology.

An overall decrease in transcript abundance was found in response to the compatible viral infection in grapevine and a greater number of repressed genes were obtained in comparison with the up-regulated group (Tables S1 and S2). A host gene shut-off is generally observed upon viral infection in animal and plants (Aranda and Maule 1998; Itaya et al. 2002). This phenomenon, associated with virus replication, is selective over a number of plant genes, while others escape from it or are induced as a result of the infection. It is notorious that the unknown biological processes constitute an important group of affected genes

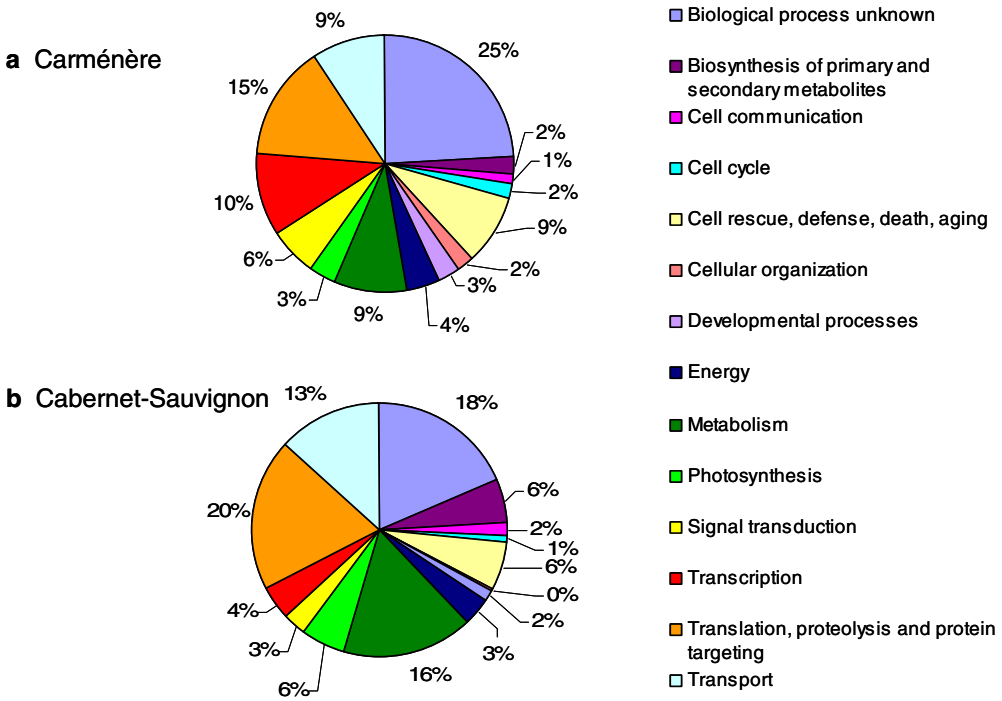

Fig. 2 Biological functions involved in grapevine response to viral infection. The figure shows the percentages of genes affected by viral disease distributed into the different biological functions for Carménère (**a**) and Cabernet-Sauvignon (**b**) cultivars. Only genes with differential expression of at least twofold were graphed, corresponding to 341 Carménère and 692 Cabernet-Sauvignon genes. To perform the biological role assignment, grapevine sequences (TCs from TIGR Grape Gene Index, (http://www.tigr.org/tdb/tgi), matching to probe sets on the array were aligned to *Arabidopsis* genome (WU-BLAST 2.0) and then all loci identifiers were used to obtain the GO annotation and functional characterization (http://www.geneontology.org)

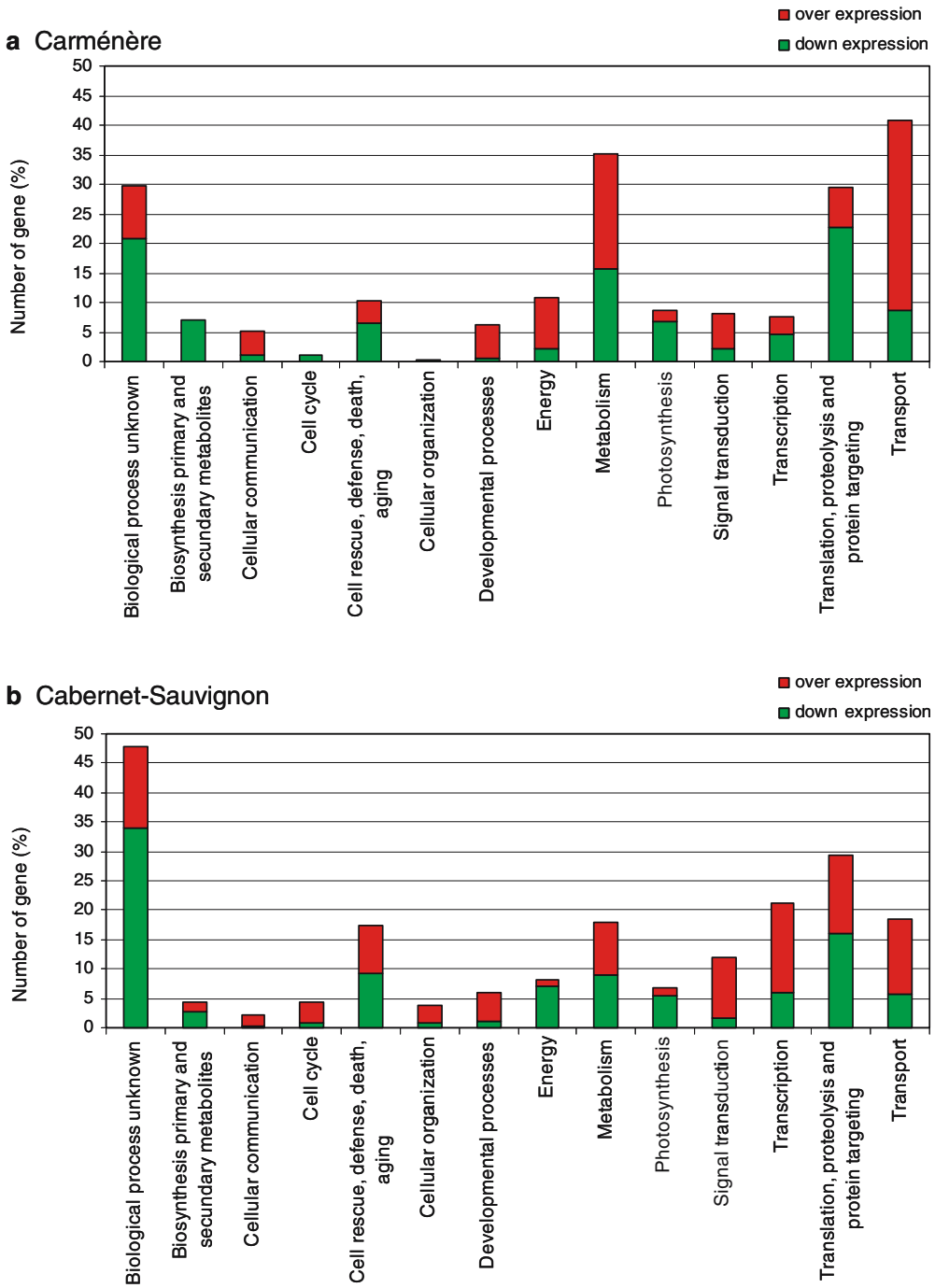

Fig. 3 Distribution of up- and down-regulated genes during viral infection into functional categories. The distribution of genes induced or repressed by viral disease in grapevine cultivars Carménère (**a**) and Cabernet-Sauvignon (**b**) are shown. Each *bar* contains all genes that fall into the same functional class, and induced and repressed genes are denoted by the *red* and *green bars*, respectively. Known and putative functions of virus-induced genes were deduced from a TBLASTX search of their DNA sequences against the non-redundant GenBank protein database. High scoring pairs with expected *E* values less than 10^{-15} were considered significant. *Red* and *green columns* represent induced and repressed genes, respectively

(Figs. 2 and 3). The low similarity of some *Vitis* gene sequences to *Arabidopsis* and others known species could explain in part the abundance of genes that belong to this category.

An important number of down-regulated genes represent protein products associated with chloroplast (Fig. 4). Specifically, genes that code for photosystem proteins and enzymes of the biosynthesis of photosynthetic pigments

Table 1 Induced genes in Carménère plants during viral infection with GLRaV-3

ID number	Description[a]	AGI number	Fold change (log2)
	Function: cell rescue, defense, death, aging		
1611350_at	Lipid transfer protein (LTP) family protein	At3g18280	3.8
1614862_at	Tropinone reductase	At2g29350	2.2
1622723_at	Harpin-induced family protein (HIN1)	At5g53730	1.8
1609152_s_at	Proline-rich family protein	At2g40820	1.5
1616495_at	Glutathione *S*-transferase	At2g30860	1.4
	Function: transport		
1613896_at	Proton-dependent oligopeptide transport (POT) family protein	At3g47960	2.7
1611326_at	Sugar transporter	At2g48020	2.4
1610422_at	SEC14 cytosolic factor family protein	At3g51670	2.3
1622455_at	Proton-dependent oligopeptide transport (POT) family protein	At2g40460	2.1
1621072_at	Purine permease	At1g28220	1.8
1614764_at	Monosaccharide transporter	At1g34580	1.4
1615966_at	Tetratricopeptide repeat (TPR)-containing protein	At2g42580	1.4
1618261_at	Proton-dependent oligopeptide transport (POT) family protein	At1g68570	1.3
1608751_at	Amino acid permease family protein	At2g34960	1.2
	Function: developmental processes		
1621255_at	No apical meristem (NAM) family protein (NAC2)	At4g17980	3.6
1613141_at	No apical meristem (NAM) family protein (NAC2)	At2g27300	2.0
1609117_at	No apical meristem (NAM) family protein (NAC2)	At5g61430	1.7
	Function: translation, proteolysis and protein targeting		
1620371_at	Aspartyl protease family protein	At3g52500	2.8
1618916_at	Cysteine synthase	At3g04940	2.2
1621663_at	Zinc finger (C3HC4-type RING finger) family protein	At3g07120	1.1
	Function: cellular organization		
1613527_at	Expansin (EXP1)	At1g69530	1.8
1619456_at	Pectinesterase family protein	At3g14310	1.7
1612339_at	Hydroxyproline-rich glycoprotein family protein	At1g21695	1.5
	Function: signal transduction		
1617163_at	Auxin efflux carrier family protein	At2g17500	1.8
1622637_at	*Arabidopsis* pde1 suppressor 1 protein (ASP1)	At2g37550	1.1
1613079_at	Tyrosine specific protein phosphatase family protein	At1g05000	1.0
1621823_at	Two-component responsive regulator/response reactor (RR4)	At3g57040	1.0
	Function: energy and photosynthesis		
1613920_at	Cytochrome P450 family protein	At3g26330	1.7
1615855_at	Cytochrome P450 71A21 (CYP71A21)	At3g48320	1.6
1617740_at	Cytochrome P450 family protein	At2g45550	1.3
1607367_at	Protein kinase family protein	At5g39020	1.2
	Function: biosynthesis and metabolism		
1621066_s_at	Glycosyl hydrolase family 1 protein	At1g02850	1.6
1611876_s_at	Acidic endochitinase (CHIB1)	At5g24090	1.3
1611876_s_at	Acidic endochitinase (CHIB1)	At5g24090	1.1
1611876_s_at	Acidic endochitinase (CHIB1)	At5g24090	1.0
	Function: transcription		
1611013_at	Zinc finger (C2H2 type) family protein	At1g34370	1.1
1617626_at	Ethylene-responsive family protein	At4g29100	1.1
	Function: biological process unknown		
1608383_at	Multi-copper oxidase type I family protein	At1g76160	2.0
1618364_at	C2 domain-containing protein	At3g17980	1.2
1612494_at	VQ motif-containing protein	At1g35830	1.1

The list include genes that are induced more than twofold in response to GLRaV-3 infection in Carménère plants. Functional categories were assigned considering sequences of genes represented on the array and their annotation (Gene Ontology Consortium)

[a]Description is based on *Arabidopsis thaliana* genes (*E* value $<10^{-15}$)

were repressed. A decrease in photosynthetic activity and chlorosis commonly occurs as a symptom of different viral diseases. In relation to this, viral systemic infection causes a depletion of the photosystem II core complex in tobacco leaves that can correlate with the chlorosis response (Lehto et al. 2003).

Grapevine plants activate a defense response against the systemic viral infections

The magnitude of gene expression changes obtained in our grapevine experiments is less dramatic than resistance-associated responses, and is in accordance with previous studies of systemic viral infections in *Arabidopsis* (Golem and Culver 2003; Ishihara et al. 2004). During an active defense response, biochemical and cell-structural changes destined to restrict the pathogen spread are induced and a systemic acquired response (SAR) mediated by salicylic acid is activated throughout the plant. Pathogenesis-related proteins (PRs), stress-related and detoxifying enzymes genes are straightly associated with this condition. In viral compatible infections, these genes vary along the infection progress (Ishihara et al. 2004) even when some of them are clearly induced (Whitham et al. 2003; Ishihara et al. 2004).

In our case, the defense, cell rescue, and aging functional group contains both up-regulated and repressed genes (Fig. 3). Thus, the host is not passive while systemic infection occurs and some grade of defense is activated, as has been observed in other systemic viral infections (O'Donnell et al. 2003). The LTP gene appears induced in infected tissues of both cultivars (Tables 1, 2 and 3). The mutant *dir1* (defective in induced resistance 1) is unable to develop SAR, and interestingly the wild-type DIR1 share sequence similarity to LTPs (Durrant and Dong 2004). Thus, this protein could be involved in the generation of a mobile signal during the SAR. LTPs were associated with hypersensitive response (HR) in pepper and necrotic viral symptoms in *Arabidopsis* (Sohal et al. 1999; Park et al. 2002), but their role in compatible interaction has not been shown before.

Beta-1, 3-glucan, or callose synthase gene (CALS1) takes part in the response against wounding and in SAR mediated by salicylic acid in *Arabidopsis* (Ostergaard et al. 2002). Callose synthesis increases during the plant defense against viruses as part of the HR. In compatible infection, callose synthesis increases, but the reaction is not enough to stop the infection (Ehrenfeld et al. 2005). In the grapevine viral infection, the enzyme beta-1, 3-glucanase that hydrolyzes callose, is also induced, as was observed in *Arabidopsis* in response to several viruses (Whitham et al. 2003) and also in Populus infected with PopMV (Smith et al. 2004).

The repression of some defense genes may be related to the prevalence of GLRaV-3 infection in the grapevine plants over several years. Lactoylglutathione lyase is involved in glutathione-based detoxification of methylglyoxal during plant responses to hormones and either salt or osmotic stresses (Singla-Pareek et al. 2003). Grapevine infected plant showed a lower expression of this gene and probably imply a lower ability to face stressing environmental factors and thus, affect the general condition of the infected plants.

Overlapping between leaf senescence and plant response to virus

It has long been proposed that some overlap exists between leaf-senescence and pathogen-defense programs, but the extent of it remains to be determined (Quirino et al. 2000). Some Carménère virus-induced genes support this overlap concept, and examples include tropinone reductase, HIN1, aspartyl protease, and the above-mentioned LTPs. Tropinone reductase encodes a short-chain alcohol dehydrogenase and has been described as the senescence-associated gene 13 (Weaver et al. 1998). The biological function of HIN1 is unknown but shares sequence identity with NDR1 protein, which is required for the HR in *Arabidopsis* against bacteria, fungi, and viruses, probably associated with cell death (Pontier et al. 1999). Aspartyl proteases have been related with protein processing at different stages of plant development, including leaf senescence (Quirino et al. 2000; Simoes and Faro 2004). Besides its role in the recycling of nutrients during senescence, aspartyl proteases are related with the degradation of PR proteins in tomato and tobacco, probably preventing an over-accumulation of them. Aspartyl proteases may also play an important role during chloroplast degradation, explaining the chlorosis symptoms and the lower photosynthetic activity observed in some virus-infected plants.

Transport is a function highly affected by viral systemic infection in grapevine

Genes associated with the membrane and endomembrane systems are expressed in both cultivars after the infection with GLRaV-3. This effect is clearly observed in Carménère cultivar (Fig. 4). Many of these genes are associated with the transport function. For example, the expression of monosaccharides, and other sugars transporters are induced. A clear induction of genes that code for sugar transporters has been reported in *Arabidopsis* and tomato in response to fungal infections (Fotopoulos et al. 2003; Garcia-Rodriguez et al. 2005). In the last case, a re-direction of sugars to regions colonized by the pathogen has been suggested. An alteration in source–sink balance could explain the delay in berries ripening and anthocyanins accumulation that accompany several

Table 2 Induced genes in Cabernet-Sauvignon plants during viral infection with GLRaV-3

ID number	Description[a]	AGI number	Fold change (log2)
	Funtion: cell rescue, defense, death, aging		
1611350_at	Lipid transfer protein (LTP) family protein	At3g18280	3.0
1611587_at	Agenet domain-containing protein	At4g17330	2.1
1610405_at	Patatin family protein	At2g39220	2.0
1621974_at	MAP kinase phosphatase (MKP1)	At3g55270	1.8
1615199_at	Cryptochrome 1 apoprotein (CRY1)	At4g08920	1.7
1608317_at	Ankyrin repeat family protein	At4g19660	1.6
1615500_at	Coronatine-insensitive 1 (FBL2)	At2g39940	1.6
1608541_s_at	Tropinone reductase	At5g06060	1.6
	Function: developmental processes		
1621723_at	No apical meristem (NAM) family protein (NAC2)	At3g15510	2.5
	Function: transcription		
1611132_at	p300/CBP acetyltransferase-related protein 2 (PCAT2)	At1g79000	2.4
1611132_at	p300/CBP acetyltransferase-related protein 2 (PCAT2)	At1g79000	2.2
1613813_a_at	Auxin-responsive factor (ARF1)	At5g62000	2.1
1613813_a_at	Auxin-responsive factor (ARF1)	At5g62000	1.9
1613366_at	TCP family transcription factor	At3g15030	1.8
1614931_at	Myb family transcription factor	At3g16350	1.8
1609115_at	Bromo-adjacent homology (BAH) domain protein	At3g48050	1.7
1609629_at	AP2 domain-containing transcription factor family protein	At5g61890	1.6
1618129_at	Basic helix-loop-helix (bHLH) protein-related	At2g31280	1.6
1607523_at	TCP family transcription factor 3 (TCP3)	At1g53230	1.6
1618544_at	Pumilio/Puf RNA-binding domain-containing protein	At3g20250	1.6
1607608_at	Squamosa promoter-binding protein-like 7 (SPL7)	At5g18830	1.6
1608789_at	Squamosa promoter-binding protein-like 1 (SPL1)	At2g47070	1.6
1617034_at	Myb family transcription factor	At2g36960	1.6
1611133_at	FF/WW domain-containing protein	At3g19670	1.6
	Function: translation, proteolysis and protein targeting		
1618030_at	40S ribosomal protein S23 (RPS23B)	At5g02960	2.2
1618901_at	Ubiquitin-conjugating enzyme family protein	At2g33770	2.0
1619941_at	CBL-interacting protein kinase 1 (CIPK1)	At3g17510	1.8
1615163_at	WD-40 repeat family protein	At1g15750	1.8
1622325_a_at	Protein kinase family	At1g12580	1.7
1607992_at	Protein kinase family	At1g67580	1.7
1617742_at	Homeodomain-containing protein	At2g35940	1.7
1621344_at	Zinc finger (C3HC4-type RING finger) family protein	At2g30580	1.6
1620968_at	F-box family protein (FBL15)	At4g33210	1.6
1614451_at	Phosphatidylinositol 3- and 4-kinase family protein	At2g17930	1.6
	Function: biosynthesis and metabolism		
1617584_s_at	Glycosyl transferase family 20 protein	At1g06410	2.2
1615939_at	Callose synthase 1 (CALS1)	At1g05570	1.9
1609749_at	2-oxoglutarate-dependent dioxygenase (AOP1.2)	At4g03070	1.7
1618666_at	Leucine-rich repeat family protein	At1g78230	1.7
1610130_at	Leucine-rich repeat family protein	At1g15740	1.6
	Function: cellular organization		
1609211_at	Octicosapeptide/Phox/Bem1p (PB1) domain protein	At2g01190	2.1
1613527_at	Expansin (EXP1)	At1g69530	1.9
1607195_at	Calcium-binding EF hand family protein	At1g20760	1.7
	Function: signal transduction		
1610480_at	Transcription activator NAC1 (NAC1)	At1g56010	2.3
1619722_at	Mitogen-activated protein kinase (MPK16)	At5g19010	1.7
1618465_at	Shaggy-related protein kinase alpha (ASK1)	At5g26751	1.7
1615909_s_at	Protein kinase family	At5g58350	1.7
	Function: cell cycle		
1612309_at	Rad21/Rec8-like family protein	At5g16270	1.7
1618868_at	Small MutS-related domain-containing protein	At2g26280	1.6

Table 2 (continued)

ID number	Description[a]	AGI number	Fold change (log2)
1618868_at	Small MutS-related Function: transport	At2g26280	1.6
1609920_at	PBS lyase HEAT-like repeat-containing protein Function: biological process unknown	At5g19820	1.6
1622668_at	F-box family protein	At3g26000	2.1
1610344_at	Zinc-binding family protein	At1g21000	1.9

[a]Description is based on *Arabidopsis thaliana* genes (E value $<10^{-15}$)
List include genes that are induced over twofold in response to GLRaV-3 infection in Carménère plants. Functional categories were assigned considering sequences of genes represented on the array and their annotation (Gene Ontology Consortium)

viral infections as a relation between these two processes has been described in grapes (Cakir et al. 2003). Additionally, it has been reported that virus-infected grapevine plants produce juice must with an important decrease in sugars and its organoleptic properties (Borgo and Angelini 2002).

A group of the induced genes corresponds to proton-dependent oligopeptide transporters (POT) (Table 1) that constitutes a family of proteins associated with membranes (Lubkowitz 2006). As POTs are involved in auxin transport, an up-regulation of this kind of protein alters the homeostasis of this hormone, which has been implicat-

Table 3 Overlapping of viral responses of grapevine cultivars Carménère and Cabernet-Sauvignon

Description[a]	AGI number (ID number)	Fold change (log2)	
		Carménère	Cabernet sauvignon
Function: cell rescue, defense, death, aging			
Lipid transfer protein (LTP) family protein	At3g18280 (1611350_at)	3.8	3.0
Tropinone reductase, putative	At2g29350 (1614862_at), At5g06060 (1608541_s_at)	2.2	1.6
Hydroxyproline-rich glycoprotein family protein	At1g21695 (1612339_at), At3g22440 (1613281_at)	1.5	1.5
Function: transport			
SEC14 cytosolic factor family protein	At3g51670 (1610422_at), At4g39170 (1612370_at)	2.3	1.1
Tetratricopeptide repeat (TPR)-containing protein	At2g42580 (1615966_at), At1g76630 (1613465_at)	1.4	1.4
Amino acid permease family protein	At2g34960 (1608751_at), At5g36940 (1613466_at)	1.2	1.3
Function: translation, proteolysis and protein targeting			
Aspartyl protease family protein	At3g52500 (1620371_at), At1g49050 (1614861_at)	2.8	1.4
Zinc finger (C3HC4-type RING finger) family protein	At3g07120 (1621663_at), At2g30580 (1621344_at), At2g37150 (1606485_a_at), At3g09770 (1617082_a_at), At3g54780 (1611089_at), At4g19670 (1617731_at), At4g34100 (1607726_at)	1.3 to 1.1	1.6 to 1.1
Function: biosynthesis			
Cysteine biosynthesis	At3g04940 (1618916_at)	2.2	1.1
Function: developmental processes			
No apical meristem (NAM) family protein (NAC2)	At2g27300 (1613141_at), At4g17980 (1621255_at), At5g61430 (1609117_at), At3g15510 (1621723_at), At1g34180 (1615036_at)	3.6 to 1.7	2.5 to 1.4
Expansin, putative (EXP1)	At1g69530 (1613527_at)	1.8	1.9
Function: metabolism and energy			
Glycosyl hydrolase family 1 protein	At1g02850 (1621066_s_at), At3g06510 (1609579_at)	1.6	1.5
Cytochrome P450 71A21, putative (CYP71A21)	At3g48320 (1615855_at)	1.6	1.1
Function: signal transduction			
Two-component responsive regulator/response reactor 4 (RR4)	At3g57040 (1621823_at), At3g16857 (1622859_at)	1.0	1.3

Table 3 (continued)

Description[a]	AGI number (ID number)	Fold change (log2)	
		Carménère	Cabernet sauvignon
Function: biological process unknown			
VQ motif-containing protein	At1g35830 (1612494_at)	1.1	1.3
Not assigned		1.8	1.5
Function: cell rescue, defense, death, aging			
Dehydration-responsive protein (RD22)	At5g25610 (1621818_at)	−4.3	−3.5
DNAJ heat shock N-terminal domain-containing protein	At2g17880 (1621230_at), At4g07990 (1622195_at), At5g21430 (1614293_at), At5g16650 (1619434_at), At2g35795 (1620227_at), At5g03030 (1620749_at), At3g47940 (1618386_at)	−2.1 to −1.3	−1.6 to −1.1
Acyl carrier family protein/ACP family protein	At5g47630 (1614903_at), At4g25050 (1617249_at)	−1.3 to −1.1	−1.3
Nucleotide diphosphate kinase II, chloroplast (NDPK2)	At5g63310 (1606713_at)	−1.4	−1.1
Function: signal transduction			
Oxidoreductase, 2OG-Fe(II) oxygenase family protein	At4g21200 (1621326_at)	−3.0	−2.5
Myb family transcription factor	At3g04030 (1618968_at), At4g09460 (1616268_x_at)	−1.5	−1.4
Leucine-rich repeat family protein	At1g73070 (1610420_at), At3g50690 (1611147_at)	−1.1	−2.1
Function: biosynthesis and metabolism			
Gibberellin 20-oxidase-related	At1g50960 (1607778_at)	−2.3	−2.3
Sterol 4-alpha-methyl-oxidase 2 (SMO2)	At1g07420 (1622130_at)	−1.4	−1.3
Lactoylglutathione lyase	At1g67280 (1609873_at), At1g80160 (1619235_at)	−1.2	−2.7
Phosphoenolpyruvate carboxykinase [ATP], putative	At4g37870 (1621034_at)	−1.2	−1.0
Hydrolase, alpha/beta fold family protein	At4g12830 (1613585_at), At5g17720 (1612289_at)	−1.1	−1.4
Oxidoreductase, zinc-binding dehydrogenase family protein	At3g15090 (1612477_at), At1g23740 (1609784_s_at)	−1.1 to −1.0	−1.1
Expressed protein	At2g21280 (1622365_at)	−1.4	−2.1
Function: photosynthesis			
Proton extrusion protein-related	At4g31040 (1612624_at)	−2.2	−2.6
Photosystem II 11 kDa protein-related	At1g05385 (1622109_at)	−2.1	−1.7
Thylakoid lumenal 17.4 kDa protein, chloroplast	At5g53490 (1606898_at)	−1.5	−1.1
Short−chain dehydrogenase/reductase (SDR) family protein	At4g20760 (1615296_at), At4g11410 (1615139_at), At4g23420 (1619126_s_at)	−1.3 to −1.0	−1.4
BolA-like family protein / Fe-S metabolism associated domain-containing protein	At4g26500 (1618897_at), At5g09830 (1613430_a_at)	−1.1	−1.2
Function: translation, proteolysis and protein targeting			
Translation initiation factor IF-1, chloroplast, putative	At4g11175 (1621593_s_at)	−2.3	−2.8
Ribosomal protein L20 family protein	At1g16740 (1611558_at)	−1.9	−2.6
Metallopeptidase M24 family protein	At4g37040 (1612727_at)	−1.4	−1.2
Zinc finger (C3HC4-type RING finger) family protein	At3g55530 (1617388_at), At5g01520 (1622027_at), At2g45530 (1614539_at)	−1.3	−1.2
Glycosyl hydrolase family 20 protein	At3g14570 (1613934_s_at), At1g65590 (1617875_at)	−1.2	−1.7
Signal peptidase I family protein	At3g24590 (1616672_at), At3g08980 (1616181_at)	−1.2	−1.2
ATP-dependent Clp protease proteolytic subunit, putative	At1g09130 (1612759_at), At1g11750 (1609882_s_at)	−1.1	−1.1
Heat shock protein 70, putative	At4g24280 (1610958_at)	−1.0	−1.0
Expressed protein	At3g45050 (1618846_at)	−1.3	−1.2
	At2g29180 (1609530_at)	−1.2	−1.2

Table 3 (continued)

Description[a]	AGI number (ID number)	Fold change (log2)	
		Carménère	Cabernet sauvignon
Function: transcription and cell cycle			
Zinc finger (C2H2 type) family protein	At5g42640 (1621309_at), At5g04240 (1609960_at)	−1.6	−2.3
DNA-directed RNA polymerase, putative	At5g60040 (1613137_at)	−1.5	−2.2
Function: transport			
SEC14 cytosolic factor, putative	At4g39170 (1612054_at)	−2.1	−1.2
Cytochrome *c* biogenesis protein family	At5g54290 (1606749_s_at), At1g22840 (1614989_at)	−1.2	−1.3
Ferredoxin-related protein	At4g32590 (1616414_at)	−1.1	−1.2
Integral membrane family protein	At3g02690 (1613501_at), At1g10030 (1615948_at)	−1.1	−1.0
Function: developmental processes			
Late embryogenesis abundant protein	At3g50790 (1614129_at)	−1.6	−1.3
Function: biological process unknown			
Transferase family protein	At5g23970 (1618389_at), At2g19070 (1614485_at)	−1.3	−2.1

[a]Description based in *Arabidopsis thaliana E* value $<10^{-15}$

Table shows genes that exhibit a similar behavior in both cultivars during infection with GLRaV-3. Genes with expression level over twofold in response to GLRaV-3 infection were considered for this analysis

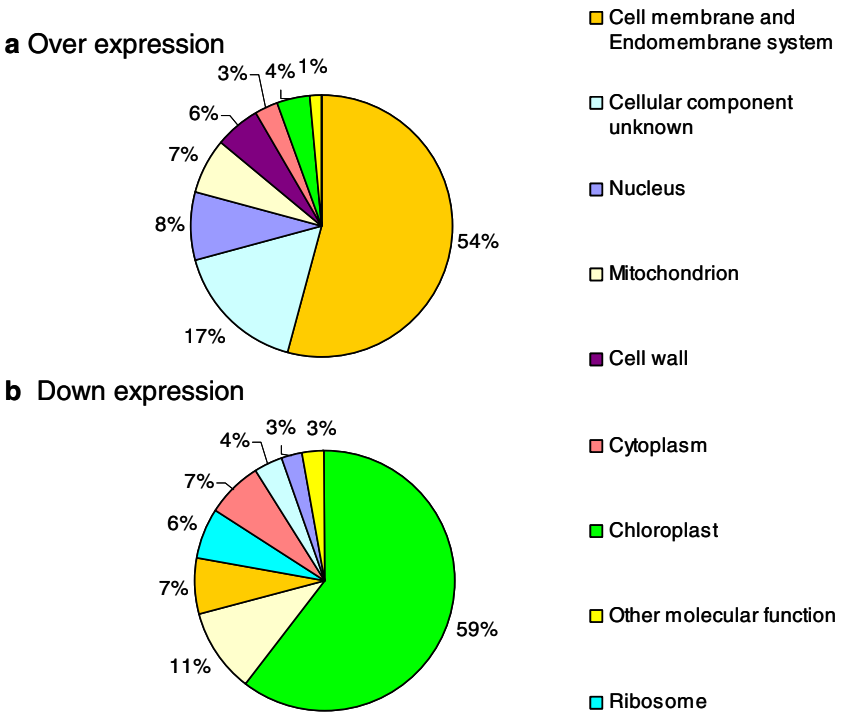

Fig. 4 Cellular component involved in grapevine cv. Carménère response to viral infection. The figure shows the percentages of induced (**a**) and repressed (**b**) genes during viral infection of Carménère grapevine plants distributed into cell localization categories. Only genes with differential expression of at least twofold were graphed, corresponding to 52 induced genes and 289 repressed genes. To carry out the cellular component assignment, grapevine sequences (TCs from TIGR Grape Gene Index, (http://www.tigr.org/tdb/tgi), matching to probe sets on the array were aligned to *Arabidopsis* genome (WU-BLAST 2.0) and then, all loci identifiers were used to obtain the GO annotation and functional characterization (http://www.geneontology.org)

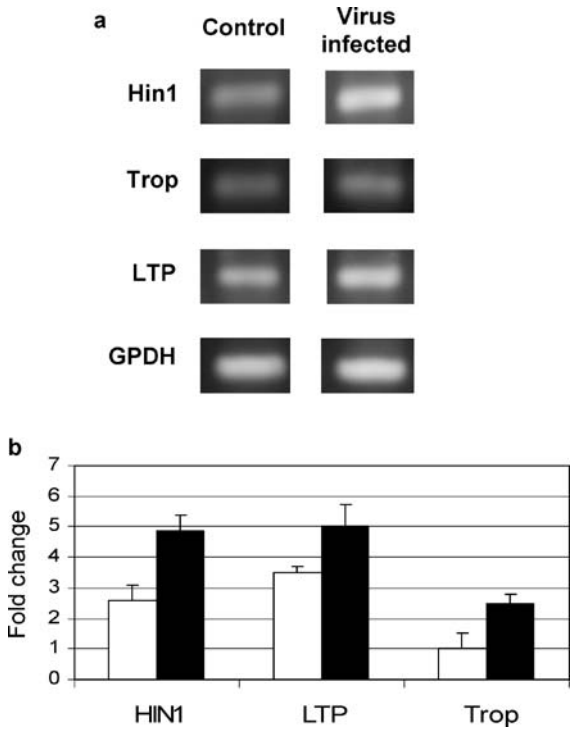

Fig. 5 Change expression of defense genes during viral infection. A verification of changes of gene expression of defense related genes in *Vitis vinifera* Carménère plants by semiquantitative RT-PCR. **a** Gene-specific primers amplify fragments of 300 bp. All amplifications were done in similar conditions. **b** Quantification of amplified products was done using Scion Image beta 4.0.2 software (Scion Corporation). Fold-change relative to a healthy control for each gene was calculated with GPDH gene as constitutive control

ed with systemic viral diseases. For example, TMV replicase interacts with PAP1 protein, a putative regulator of auxin response, interfering with the hormone activity in *Arabidopsis* and probably participating in the induction of specific disease symptoms (Padmanabhan et al. 2005). Furthermore, it has been suggested that the plant defense response during infection with viruses could be regulated through auxin homeostasis (Mayda et al. 2000), and viruses can alter the steady state of this hormone, leading to morphogenetic alterations, such as the leaf distortion characteristic of leaf roll disease (Pennazio and Roggero 1996). In agreement with this, the up-regulation of an auxin efflux carrier protein was observed in virus-infected Carménère plants.

Host genes involved in viral cycle

A successful viral infection requires the completion of events, such as viral genome replication, cell-to-cell movement, and long-distance transport. Some host genes have been implicated in these processes, and, in this sense, the repression of two DEAD/DEAH box RNA helicase and DNAJ heat shock protein is intriguing. It has been demonstrated that the DEAD-box RNA helicase is required for Brome Mosaic Virus (BMV) RNA synthesis (Maule et al. 2002). DNAJ heat shock protein acts as a chaperone and it has been shown to participate in the negative-strand RNA synthesis of BMV (Tomita et al. 2003). Replication mechanism of GLRaV-3, as well as the other members of the Closteroviridae family, involves the generation of multiple subgenomic RNAs through the negative-strand RNA synthesis (Martelli et al. 2002). Thus, a plant's response to viral infection could occur by a lower expression of DEAD/DEAH box RNA helicase and DNAJ heat shock protein, to attempt to counteract viral replication.

Another important aspect related to infection is viral movement. It was demonstrated that pectin methylesterase (PME) is necessary for cell-to-cell and systemic movement of TMV in tobacco plants (Chen and Citovsky 2003; Chen et al. 2000). A PME gene is induced in infected Carménère plants. PME role in viral spread of TMV could be derived from its direct interaction with the tobamovirus movement protein. Although none of the ORFs described in GRLaV-3 genome has been assigned as a movement protein, it is possible that some ORFs with unknown functions may act in viral spread (Ling et al. 2004).

In GLRaV-3-infected grapevine plants we observed a repression of the expression of the heat shock protein 70, Hsp70 gene, which contrasts with its induction in other viral infections (Maule et al. 2002; Whitham et al. 2003; Aparicio et al. 2005). Members of the Closteroviridae family encode a Hsp70 homologue (Hsp70h) that is necessary for virion assembly and for cell-to-cell movement of a closterovirus (Alzhanova et al. 2001). Hsp70h, located at plasmodesmata, could, therefore, be participating in virion translocation and acting as a chaperone or supplying energy through the Hsp70h-mediated ATP hydrolysis.

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