Plant Mol Biol (2011) 77:261–274
DOI 10.1007/s11103-011-9807-8

Compatible GLRaV-3 viral infections affect berry ripening decreasing sugar accumulation and anthocyanin biosynthesis in *Vitis vinifera*

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Received: 22 February 2011 / Accepted: 6 July 2011 / Published online: 24 July 2011

Abstract Virus infections in grapevine cause important economic losses and affect fruit quality worldwide. Although the phenotypic symptoms associated to viral infections have been described, the molecular plant response triggered by virus infection is still poorly understood in *Vitis vinifera*. As a first step to understand the fruit changes and mechanisms involved in the compatible grapevine-virus interaction, we analyzed the berry transcriptome in two stages of development in the red wine cultivar Cabernet Sauvignon infected with Grapevine leafroll-associated virus-3 (GLRaV-3). Analysis of global gene expression patterns indicate incomplete berry maturation in infected berries as compared to uninfected fruit suggesting viral infection interrupts the normal berry maturation process. Genes with altered expression in berries harvested from GLRaV-3-infected vines as compared to uninfected tissue include anthocyanin biosynthesis and sugar metabolism genes. The reduction in transcript accumulation for sugar and anthocyanin metabolism during fruit development is consistent with a dramatic reduction in anthocyanin biosynthesis as well as reduced sugar levels in berries, a hallmark phenotypic change observed in virus infected grapevines. Analysis of key regulatory factors provides a mechanism for the observed gene expression changes. Our results provide insight into commonly observed phenotypic alterations in virus infected vines and the molecular mechanisms associated with the plant response to the virus during berry ripening.

Keywords Virus infection · Berry maturation · Affymetrix · Anthocyanins · Sugar metabolism

Electronic supplementary material The online version of this article (doi:10.1007/s11103-011-9807-8) contains supplementary material, which is available to authorized users.

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Introduction

Economically, *Vitis vinifera* is one of the most important planted fruit species in the world for fresh consumption and wine production. Unfortunately, grapevine cultures present significant decrease in yield due to biotic stresses. Among them, viral infections are difficult to control producing an important impact in grapevine physiology, causing significant economic losses every year (Martelli and Walter 1998). In compatible plant-virus interactions, pathogens spread through all plant tissues without inducing a resistance response, generating global cellular stress and developmental defects (Whitham et al. 2006). Albeit unable to stop viral replication and systemic infection, susceptible hosts are not passive against viruses. The plant

response involve changes in the expression of defense and stress-associated genes (Whitham et al. 2003; Shimizu et al. 2007; Babu et al. 2008).

In grapevine, more than 58 different viruses have been described, many of which have a negative impact on the plant physiology (Martelli and Boudon-Padieu 2006). Among them, Grapevine leaf-roll-associated virus-3 (GLRaV-3) is one of the most widely spread viruses in the world. GLRaV-3 belongs to the genus *Ampelovirus* of the family *Closteroviridae* and is a positive single-strand RNA virus transmitted by grafting and mealy bugs (Ling et al. 2004). Different phenotypic symptoms have been associated with the disease caused by this virus, including leaf deformations and alterations in fruit quality aspects such as color and taste (Martelli 1993; Borgo and Angelini 2002). However, little is known regarding the molecular mechanisms underlying these alterations. A previous work characterized the compatible infection of GLRaV-3 in grapevine plants (Espinoza et al. 2007b) and up-regulation of sugar transporters and senescence-related gene expression have been observed in GLRaV-3 infected leaves (Espinoza et al. 2007a, b). Nevertheless, the virus-associated molecular changes during fruit ripening have not been analyzed.

Berry development has been described as happening in two growing phases separated by a transitory lag period (Coombe 1992). Stage I corresponds to a period of fast cell division and elongation with rapid accumulation of organic acids. Stage II is mainly characterized by a slow growth rate that precedes ripening. The transition from berry growth to berry ripening is known as veraison and begins at the end of Stage II. During ripening, significant changes occur in the fruit both at the level of gene expression as well as physiology and structure: the cell wall softens, pericarpic cells are expanded and sugars and anthocyanins accumulate (Terrier et al. 2005; Deluc et al. 2007; Lund et al. 2008). These changes contribute to the quality characteristics expected of a ripe fruit such as softness, size, sweetness and dark color in the case of black and red grapes.

We investigated the effect of GLRaV-3 viral infection during berry ripening in Cabernet Sauvignon grapevine. We analyzed global gene expression patterns during fruit development in GLRaV-3 infected and uninfected grapevines. We observed several physiological and developmental changes in berries from infected vines as compared to fruits from uninfected vines. The global gene expression pattern of fruits from infected vines showed some of the characteristics of immature fruits of a virus-free vine. Many genes associated with anthocyanin biosynthesis and sugar metabolism were down regulated in berries from infected vines as compared to uninfected fruit. Analysis of key metabolic genes and their cognate transcription factors provide insight into the molecular changes that may explain altered levels of sugar and anthocyanins metabolites.

Materials and methods

Plant material and virus detection

Six-years-old uninfected and GLRaV-3-infected *V. vinifera* cv. Cabernet Sauvignon grapevines were obtained from the nursery of the Facultad de Agronomía e Ingeniería Forestal, Pontificia Universidad Católica de Chile. Four uninfected and three virus-infected vines were maintained separately in the field under similar growing conditions in the same experimental block. Uninfected plants in this study were defined as plants without any detectable levels of the following thirteen viruses as determined by RT-PCR: Grapevine virus A (GVA), Grapevine virus B (GVB), Grapevine fanleaf virus (GFLV), Grapevine fleck virus (GFkV), Tomato ringspot virus (ToRSV), Grapevine rootstock stem lesion associated virus (GRSLaV) and Grapevine leaf-roll-associated viruses (GLRaV) 1, 2, 3, 4, 5, 6 and 7. These viruses were chosen because they have a high infectious incidence in Chile. Optimal PCR primers (Table S1) for amplification of each virus were designed using MUMER software (Kurtz et al. 2004). Reverse transcriptase reactions were carried out as described below, while PCR reactions were performed as described in Matus et al. (2008). In addition, all uninfected plants were free of bacterial, fungal or viroids disease symptoms.

Gene expression analysis, sugar and phenolic compounds characterization (see below) of berries harvested from uninfected and virus-infected grapevines were carried out at four developmental stages, according to Coombe (1995). Grapevine berries were harvested at 4 weeks intervals throughout fruit development, corresponding to immature berries [E-L stage 31, 4 weeks after flowering (WAF)] and three time points within Stage III (E-L stages 35, 36 and 38), spanning from veraison to harvest (8, 12 and 16 WAF, respectively). The veraison state was considered when 30–50% of the berries in the bunch presented a colored phenotype. Two hundred fifty berries (3–4 clusters, from north side of the vine) of each selected plant were collected in the abovementioned stages. In the case of veraison, similar quantity of color and colorless berries were pooled. All plant tissues were frozen in liquid nitrogen immediately upon collection in the field and stored at −80°C until analyzed.

Optical and transmission electronic microscopy (TEM)

Leaves and immature berries clusters from uninfected and virus-infected vines were prepared for optical and transmission electron microscopy. For optical analysis, samples were fixed, prepared and stained with Safranin and Fast Green as described previously (Johansen 1940). Sections

were observed and photographed using a Nikon E800 Eclipse Optical Microscope.

For TEM analysis, samples were fixed in cacodylate buffer 1% (pH 7.2) and 3% glutaraldehyde for 3 h at room temperature (Lartey et al. 1997). Thin sections (70–80 nm) were obtained using a Sorvall MT2-B ultramicrotome, stained with uranyl acetate (Epstein and Holt 1963) followed by lead citrate (Reynolds 1963) and examined in a Philips Tecnai 12 transmission electron microscope.

Gene expression analysis

Total RNA was extracted from both uninfected and virus-infected *V. vinifera* leaf and entire berries, following the Tris–LiCl method modified by da Silva et al. (2005). RNA samples for microarray hybridizations were further quantified and analyzed by microfluidic analysis employing the Agilent Technologies' 2100 Bioanalyzer. RNA samples used for RT-qPCR were quantified using RiboGreen and were reverse transcribed using the Superscript II reverse transcriptase and random hexamers (Invitrogen). The obtained cDNA was diluted fivefold and used in qPCR experiments.

For microarray hybridization, seven *V. vinifera* vines (four uninfected and three infected) were selected for global gene expression analysis. The mRNA expression profiles of two berry developmental stages (E-L stage 35 and 38) were compared using the Affymetrix GeneChip® Vitis genome array, version 1.0. RNA (6.5 µg) samples were processed with the GeneChip® Expression 3′-Amplification Reagents One-cycle cDNA synthesis kit (Affymetrix) according to the manufacturer's instructions. The fragmented cRNA (10 µg) was hybridized on a *V. vinifera* GeneChip® genome array using standard procedures (45°C for 16 h). The arrays were washed and stained in a Fluidics Station 450 (Affymetrix). Array scanning was carried out with the GeneChip® scanner 300 and image analysis was performed using the GeneChip® Operating Software. Thereafter, GeneChip® array data were quality assessed using a set of standard quality control steps described in the Affymetrix manual "GeneChip® Expression Analysis: Data Analysis Fundamentals". Array data were processed and normalized with RMA (Robust Multi-Array Average) (Irizarry et al. 2003) using the affy R package (Gautier et al. 2004). Approximately 69–74% of probe sets were significantly detected in all microarray hybridizations. To evaluate array reproducibility, Spearman rank coefficients were computed and ranged between 0.993 and 0.999. The raw data for all hybridizations can be found in PLEXdb (http://www.plexdb.org) (Wise et al. 2007) under VV28 experiment identifier.

To determine which genes were differentially expressed in response to the virus infection at the two berry developmental stages, a resampling-based multiple hypothesis testing (Pollard and Laan 2004) was performed on the RMA expression values, applying a multiple-testing correction (Benjamini and Hochberg 1995) ($P < 0.05$). To identify genes showing a similar expression pattern, average-linkage hierarchical clustering was performed using the Cluster 2.11 software as described previously (Eisen et al. 1998). In addition, to identify genes differentially expressed between two conditions (e.g. the two developmental stages or infected and control samples) the Rank-Product method was used ($P < 0.05$) (Breitling et al. 2004).

To improve the annotation of the *V. vinifera* GeneChip® array, all target sequences that were used as templates for the probe sets (http://www.affymetrix.org) were compared to the gene models of the *V. vinifera* genome database (Grape Genome Browser http://www.genoscope.cns.fr) using BLASTN version 2.2.15 (e value <1e−45). For functional annotation using Gene Ontology, a BLAST analysis (BLASTP, version 2.2.15, e value <1e−10) of the protein database from the *V. vinifera* genome was conducted against the protein dataset from the *Arabidopsis thaliana* genome (http://www.tair.org). In order to determine which GO terms were statistically overrepresented, we use the BioMaps tool in VirtualPlant platform (http://www.virtualplant.org) (Katari et al. 2010) with a *P* value cutoff of 0.01.

Transcript quantification was achieved using the Sensi-MixPlus SYBR Green kit (25 µl reactions; Quantace) and the Mx3000P detection system (Stratagene) using the MxPro software (version 4.01), as described in each manufacturer's manual. For the purpose of absolute quantification, RT-qPCR products for each gene of interest were obtained, cloned into the pGEM-T vector (Promega) and sequenced to confirm their identity (data not shown). The same procedure was employed for *GPDH* (XM_002263109) and *EF1-α* (GU585871), references genes used as an internal control for normalization (geNorm method, Vandesompele et al. 2002) (Table S2). Each plasmid concentration was obtained by interpolation of the obtained C_q value within the pGEM-T vector standard curve. The amplification efficiency (E) of this qPCR reaction (E = 101.5%) was calculated according to the equation $E = [10^{(-1/\text{slope})}] - 1$. Finally, several tenfold serial dilutions of each quantified plasmid were used as templates of known concentration for qPCR reactions using primers designed against the sequence of each cloned gene (Table S2). The corresponding concentration of each standard was converted into copies by using the Avogadro constant (6.023×10^{23} molecules mole^{-1}) and its molecular weight. Primer sequences, predicted Tm values, amplicon length, efficiency values for each efficiency curve are indicated in Table S2, as recommended by Bustin (2010).

Total RT-qPCR reaction volume was 25 µl, containing 2.0 µl of cDNA template and 140 nM of each primer. The reactions were performed under the following conditions: 95°C for 10 min, followed by 40 cycles of 94°C, 30 s; 55°C, 30 s; and 72°C, 30 s, followed by the melting curve analysis from 55 to 95°C. C_q values were acquired during the annealing period of the RT-qPCR. All experiments were performed with three biological replicates, with their corresponding three technical replicates.

Sugar quantification

Free sugars profiles were determined by HPLC. The chromatographic separation was achieved with a Eurospher 100-5 NH2 column (4.6 × 250 mm, 5 mm, Knauer) operating at 35°C (7971R Grace oven). The mobile phase used was acetonitrile/deionized water, 7:3 (v/v) at a flow rate of 1 ml min^{-1}, and the injection volume was 20 µl. The results are expressed in g l^{-1}, calculated by internal standard normalization of the chromatographic peak area. Sugar identification was made by comparing the relative retention times of sample peaks with standards (Sigma).

Flavonoid quantification

Berry phenolics were extracted from berry tissue (n = 60), including skin, pulp and seed as described by Venencie et al. (1997) with modifications (Peña-Neira et al. 2007). Each sample (150 µl) was injected into the HPLC–DAD for the analysis of anthocyanin compounds (Peña-Neira et al. 2007). Detection of anthocyanins was carried out scanning from $\lambda = 210$ to 600 nm. Two mobile phases were employed for elution: A: water/acetic acid (98:2) and B: water/acetonitrile/acetic acid (78:20:2). The gradient profile was 0–55 min, 100–20% A; 55–70 min, 20–10% A; 70–90 min, 10–0% A. Detection was performed by scanning from $\lambda = 210$ to 360 nm, with an acquisition speed of 1 s.

Non-anthocyanin compounds were extracted from an aliquot (50 ml) of macerated entire berries and analyzed by injecting each sample (20 µl) into a HPLC–DAD and HPLC–DAD–MS, as described by Peña-Neira et al. (2000; 2004). The identification of the different compounds was carried out by comparison of their spectra and retention times with those obtained by Peña-Neira et al. (2004; 2007). Standards (Sigma) were used in calibration curves from $\lambda = 280$ to 520 nm, by injection of different volumes of standard solutions under the same conditions as for the samples analyzed.

Statistical analysis

Gene expression profile data (RT-qPCR) and metabolic composition were statistically analyzed by two-way ANOVA to test the significance of the effects of treatments at the different stages of berry ripening ($P < 0.05$). When differences in the means were significant, Tukey post-hoc analysis was performed ($P < 0.05$).

Results

GLRaV-3 infects grapevine fruits

In order to evaluate the effect of GLRaV-3 viral infections during berry ripening, we analyzed uninfected and virus infected grapevines grown in the field. Total RNA was obtained from leaves and entire fruit organs of plants from independent plots, and the presence of 13 different viruses was evaluated using RT-PCR (see list of viruses tested in the "Materials and methods" section). The viruses selected for analysis are the most important and commonly found viral pathogens in Chilean and other country vineyards (Fiore et al. 2008; Matus et al. 2008; Coetzee et al. 2010). This analysis identified vines infected with GLRaV-3 but negative for all the other viruses tested. In addition, the group of GLRaV-3 vines was analyzed for and did not show any phenotype of bacterial, viroids or fungal diseases. GLRaV-3 infected vines showed characteristic phenotypes associated to this virus, such as red color and deformation of leaves and altered color and low sweetness of berries as compared to uninfected vines which showed normal leaf and fruit development. The group of uninfected plants was negative for all the viruses analyzed by RT-PCR (Supplemental Fig. S1, see "Methods"). Representative infected and uninfected vines are shown in Fig. 1a and b, respectively.

Quantification of GLRaV-3 RNA in infected leaves and berries was carried out by quantitative real time RT-PCR (RT-qPCR, Fig. 1c). Viral RNA levels were high in the leaves as expected based on previous studies in our group (Espinoza et al. 2007b). Surprisingly, we detected GLRaV-3 RNA in the berry (Fig. 1c). In addition, GLRaV-3 RNA levels increased during berry ripening, with the highest levels observed at E-L38 (16 weeks after flowering, WAF) ($P < 0.05$) (Fig. 1c). The presence of GLRaV-3 RNA in berries suggested the presence of viral particles in the fruit, probably colonizing the organ through the vasculature during fruit development. This virus has been described restrictive to the vascular tissues in grapevine (Martelli and Boudon-Padieu 2006). According to this idea, we observed

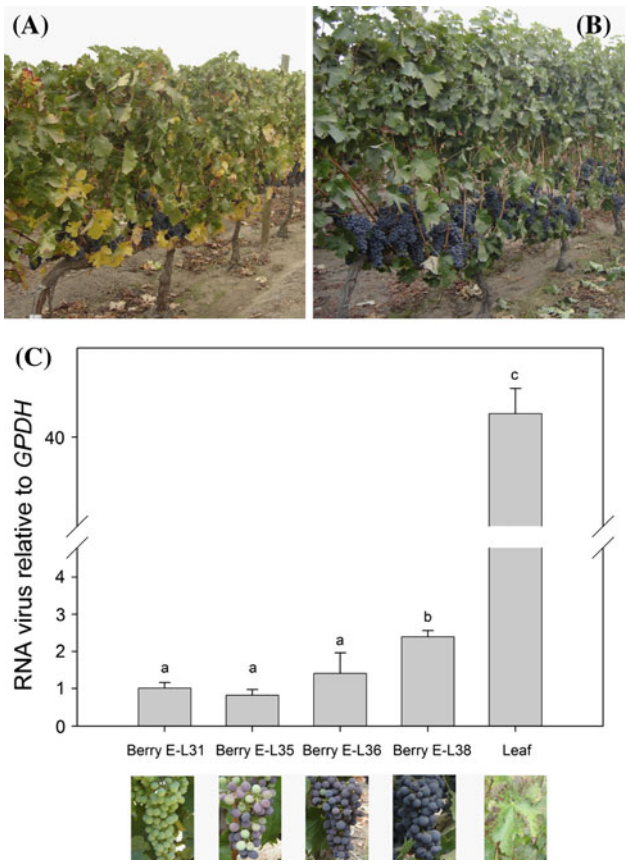


Fig. 1 Detection of viral infection in *V. vinifera* cv. Cabernet Sauvignon vines. The figure shows representative phenotypes of **a** GLRaV-3 infected grapevines and **b** uninfected vines. **c** Quantification of virus in berries (E-L stages 31–38) and leaf. The detection and relative quantification of RNA particles from GLRaV-3 infection in leaf and berries throughout ripening was carried out by RT-qPCR, amplifying a 100 bp PCR amplicon (see Table S2). Photographs depicted at the *bottom* illustrate the representative phenotype from each plant tissue, as well as the developmental stages according to Coombe (1995). Values plotted represent the mean ± standard deviation

some like viral particles in berry phloem cells of only infected plants with similar characteristic described for GLRaV-3 (Orecchia et al. 2008; Cogotzi et al. 2009), using TEM (Supplemental Fig. S1).

Global berry transcript profile indicates incomplete fruit development in vines infected with GLRaV-3

To understand the molecular changes occurring in the berries from GLRaV-3 infected vines we analyzed the fruit transcriptome at two developmental stages (E-L35: veraison and E-L38: ripening) using the *V. vinifera* Affymetrix GeneChip® 1.0. We chose these developmental stages because they are known critical points of berry development; veraison (E-L35) exhibits dramatic changes in global gene expression pattern as compared to other developmental stages and ripening (E-L38) represents the culmination of the berry developmental process (Terrier et al. 2005; Deluc et al. 2007; Lund et al. 2008). To identify differentially expressed genes, we used analysis of variance (ANOVA) implemented in the multtest R package (Pollard and Laan, 2004). A set of 3,452 genes showed significant differences in gene expression either during berry development or due to the viral infection ($P < 0.05$). We used average-linkage hierarchical clustering analysis to classify the global gene expression patterns (see Supplemental Fig. S2). This analysis identified 12 clusters representing individual groups of genes that share similar expression profiles (correlation coefficient ≥0.9) during ripening and in response to the virus infection. As expected, a large number of genes in this set (401) changed expression levels when comparing veraison (E-L35) and ripening (E-L38) in uninfected plants (Fig. 2; Table S3). Interestingly, only 43% of these genes were differentially expressed in infected berries when comparing these two developmental stages (Fig. 2; Table S4). Although grapevine is chronically infected by GLRaV-3, the virus had a greater impact at ripening than at veraison as measured by the number of differentially expressed genes. Forty-one genes were induced and 14 were repressed in berries from infected grapevine at veraison (E-L35), as compared with virus-free tissue (Table S5). In contrast, 146 genes were up-regulated and 86 were down-regulated due to the viral infection at the ripening stage (E-L38) (Table S6). Together, these results indicate that the virus affects the normal fruit ripening process, resulting in incomplete berry ripening in terms of gene expression patterns (Fig. 2).

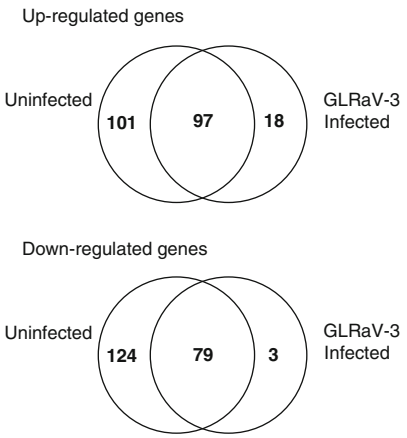


Fig. 2 Changes in gene expression in berries from uninfected and GLRaV-3-infected grapevine at ripening stage (E-L38). The figure shows Venn diagrams of up-regulated and down-regulated genes at ripening stage (E-L38) in uninfected and infected-berries. A total of 401 genes changed their expression in uninfected vines, comparing berries at veraison and ripening stages (198 up-regulated and 203 down-regulated). Only 197 genes changed their expression in virus-infected berries, comparing the abovementioned stages (115 were induced and 82 were repressed)

Viral infection affects sugar metabolites in infected berries

To gain insight into the biological functions altered by the viral infection, we analyzed the gene ontology functional annotations represented among the differentially expressed genes using the BioMaps tool in VirtualPlant (Katari et al. 2010). This analysis showed a wide range of biological processes affected in both veraison (E-L35) and ripening (E-L38) due to the GLRaV-3 infection (Fig. 3). Cell rescue, defense, death and aging were mostly induced by the virus infection, specifically over-represented during the ripening (E-L38) stage ($P < 0.01$). Several genes belonging to these biological functions have been described in other plant virus infections, suggesting a defense response in this compatible plant-pathogen interaction (Table 1). Interestingly, a set of genes altered by GLRaV-3 in infected berries is implicated in sugar metabolism and transport. This is a relevant observation since during berry ripening, sugar metabolism and accumulation are key processes that contributes to fruit quality. For this reason, a quantitative analysis of the transcript profiles for five hexose transporters and their regulatory transcription factors was carried out in four developmental stages by RT-qPCR. Interestingly, *VvHT1* and its transcription factor MSA mRNA levels were significantly repressed in virus-infected vines at most developmental stages analyzed (Fig. 4a). In addition, other sugar transporters were affected in infected berries. Furthermore, selected sugar metabolites were measured in the same samples. Consistent with the decreased expression of these genes, glucose and fructose accumulation was reduced during ripening in virus-infected vines (Fig. 4b). Accordingly, GLRaV-3 infected berries were significantly reduced in mass in comparison to uninfected tissue (Table S7). These results suggest that virus infection alters sugar levels in the fruit, possibly affecting sugar transport and its metabolism in the fruit.

Viral infection affects flavonoid metabolites in grapevine berries

Other biological functions altered by the viral infection were metabolism and biosynthesis of primary and secondary metabolites, specifically over-represented during ripening (E-L38) and not during veraison (E-L35) stages ($P < 0.01$) (Fig. 3). For example, we found the phenylpropanoid pathway to be affected by viral infection in a consistent way. Many genes from the phenylpropanoid pathway were found in cluster VIII of the hierarchical clustering analysis performed above, a group of genes repressed by the viral infection during ripening (Supplemental Fig. S2). This result suggests that the viral infection alters normal fruit metabolic processes, including the

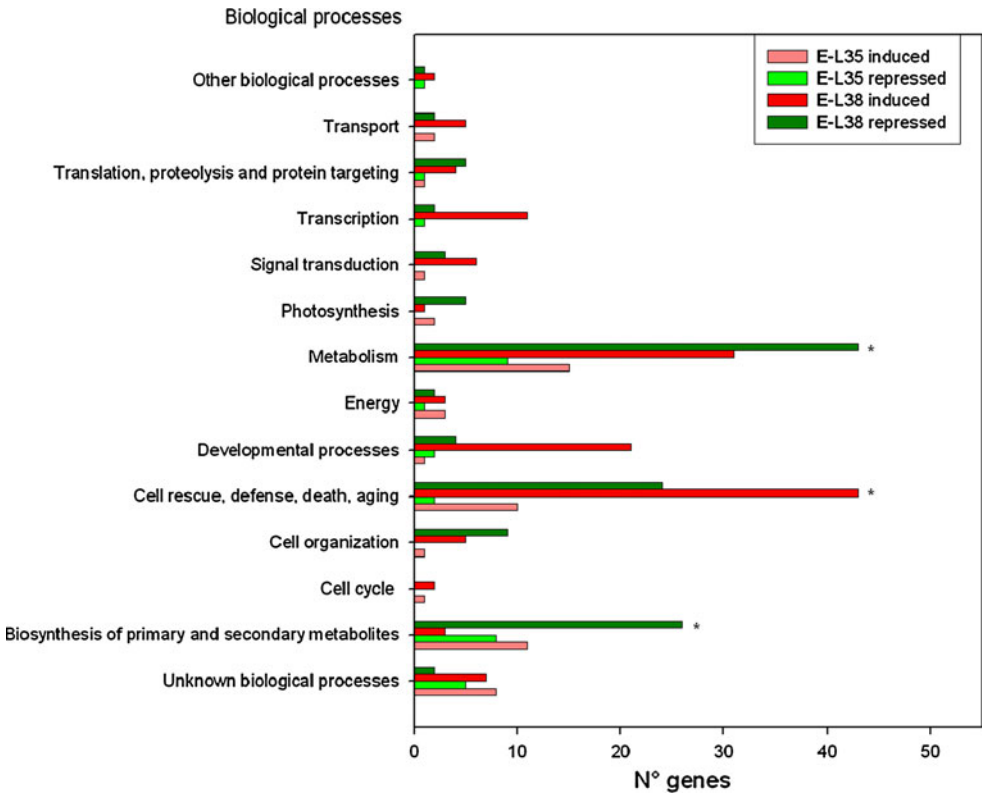


Fig. 3 Biological functions affected in berries from infected grapevine at veraison (E-L35) and ripening (E-L38) stages. The figure shows the number of genes induced or repressed in virus-infected tissue relative to uninfected tissue and its distribution into the different Gene Ontology biological functions for veraison (E-L stage 35) and ripening (E-L stage 38). Only those genes with differential expression ($P < 0.05$) were plotted, corresponding to 115 and 906 genes in veraison and ripening, respectively. Over represented categories of induced or repressed genes compared to the entire set on the *V. vinifera* genome array are indicated with asterisks ($P < 0.01$)

Table 1 Defense-related genes induced in berries from GLRaV-3-infected plants at ripening (E-L38)

ID number	Description[a]	ID genome *Vitis*[b]	AGI number[a]	Fold change (log2)	*P* value
1607895_at	CYCT1;4 (cyclin-dependent protein kinase)	GSVIVT00019948001	AT4G19600	1.43	0.01
1608058_at	Identical to E3 SUMO-protein ligase SIZ1 (SIZ1)	GSVIVT00024500001	AT5G60410	1.30	0.05
1622821_at	SOBER1 (suppressor of avrbst-elicited resistance 1); carboxylesterase	GSVIVT00011724001	AT4G22300	1.23	0.01
1619150_at	DCL1 (dicer-like1)	GSVIVT00026189001	AT1G01040	1.21	0.02
1617444_s_at	LOX2 (lipoxygenase 2)	GSVIVT00024672001	AT3G45140	1.03	0.03
1619918_at	ATCAT6/CAT6 (cationic amino acid transporter 6)	GSVIVT00013821001	AT5G04770	1.00	0.03
1614506_at	PATATIN-LIKE protein 2 (phospholipase A 2A)	GSVIVT00014789001	AT2G26560	0.97	0.05
1610756_at	Leucine-rich repeat family protein (LRR)	GSVIVT00024648001	AT3G20820	0.90	0.05
1608318_at	Receptor serine/threonine kinase, similar PR5-like	GSVIVT00011346001	AT4G18250	0.80	0.05
1608976_at	EIN2 (ethylene insensitive 2), transporter	GSVIVT00023209001	AT5G03280	0.80	0.05

The table contains defense-related genes from *V. vinifera* that change their transcript levels in response to the infection caused by GLRaV-3, compared with uninfected tissue (see "Methods")

[a] Description based on the *A. thaliana* genome, deduced from a BLASTP analysis (e value <1e−10, see "Methods")

[b] Gene models from the *V. vinifera* genome were deduced from a BLASTN analysis (e value <1e−45, see "Methods")

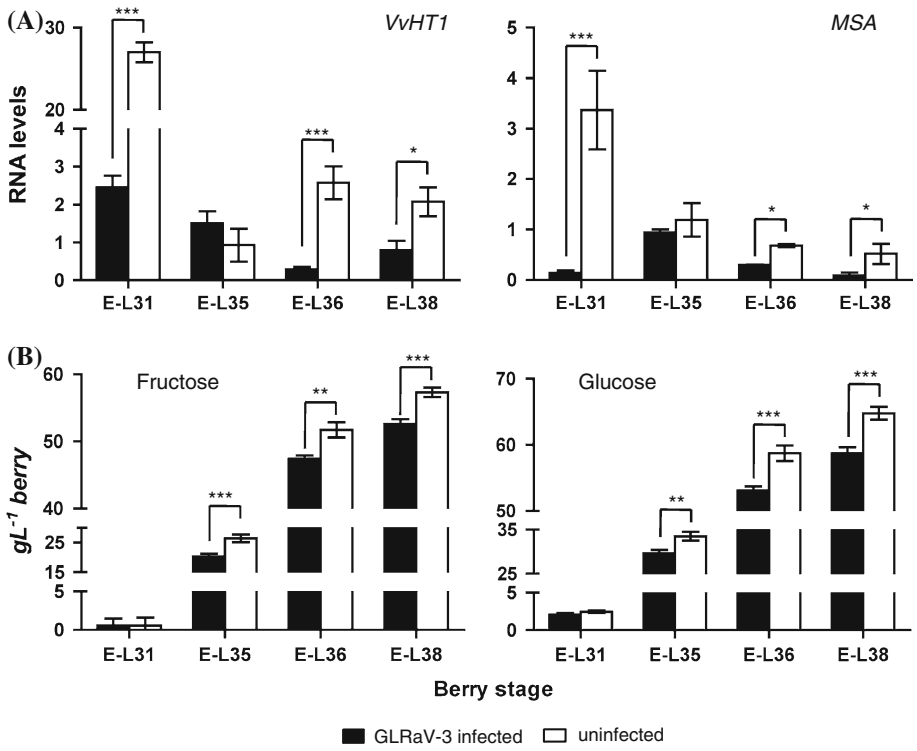


Fig. 4 Changes in mRNA levels of genes associated with sugar transport and concentration in response to virus infection at four berry developmental stages. The figure shows the transcript abundance of **a** the hexose transporter *VvHT1* and its transcriptional regulator *MSA* and **b** glucose and fructose concentrations at four ripening stages in uninfected (*white bar*) and virus-infected (*black bar*) grapevines. Veraison (E-L35) corresponds to 8 WAF. Each graph represents the mean ± standard deviation of three biological replicates. Statistical significance is marked by *one asterisk* ($P < 0.05$), *two asterisk* ($P < 0.01$) and *three asterisk* ($P < 0.001$)

phenylpropanoid pathway, with a stronger impact observed in the ripe berries.

Fruit coloring is an important fruit quality trait. The color modifications observed during the berry ripening processes are associated to the phenylpropanoid pathway (Coombe 1992). Our global gene expression analysis suggested that changes in fruit color in the infected berries could be due to alterations in the expression of genes related to the phenylpropanoid biosynthesis pathway (Supplemental Fig. S2, cluster VIII). Key genes of the anthocyanin biosynthesis pathway were identified as down-regulated in infected tissues during ripening (Table S5, S6). We validated the gene expression changes for these genes using RT-qPCR. In addition, we included transcription factors that are known to regulate the phenylpropanoid pathway throughout berry development. Figure 5a shows

mRNA level changes for some of the most important genes associated to the initial and last steps of the phenylpropanoid pathway as well as associated regulatory transcription factors at four different fruit developmental stages (Fig. 1c). These results show that the viral infection affects the transcript abundance of key phenylpropanoid biosynthetic genes validating our global gene expression analysis. For instance, *CHS2* mRNA levels were up-regulated in immature (E-L31) infected berries but repressed at the ripening stage (E-L38, Fig. 5a). The expression of the *UFGT* gene was strongly repressed by the virus at ripening (E-L38) in infected-berries as compared with uninfected berries (Fig. 5a). Furthermore, the *MYBPA1* and *MYBA* transcription factors were also affected by the virus infection (Fig. 5a). The expression levels for these regulatory factors were consistent with the observed levels of their corresponding target genes. The *MYBPA1* mRNA levels were induced in virus-infected vines before veraison (stages E-L31 and E-L35), but also repressed at berry ripening (E-L38) in infected plants. The expression level of *MYBA* was lower in infected as compared to uninfected berries in E-L36 and E-L38.

To evaluate whether gene expression changes had an impact at the metabolite level, metabolites were measured by HPLC in uninfected and GLRaV-3 infected vines, at the same berry developmental stages used for gene expression analysis. As shown in Figs. 5b, the altered expression of key metabolic and regulatory genes due to the virus infection was consistent with the observed changes of several flavonoid metabolites. Total anthocyanin accumulation was significantly lower in virus-infected berries as compared to uninfected vines (Fig. 5b). In both viral-infected and uninfected berries, no differences were observed in the anthocyanin content at veraison (E-L35). However, the anthocyanin content observed at ripening (E-L38) was 40% lower in virus-infected berries as compared to uninfected berries, concomitant with the gene expression alterations. Figure 5b shows the accumulation profile for the main anthocyanin metabolite in Cabernet Sauvignon (malvidin-3-O-glucoside, Mv3G), which resembles the

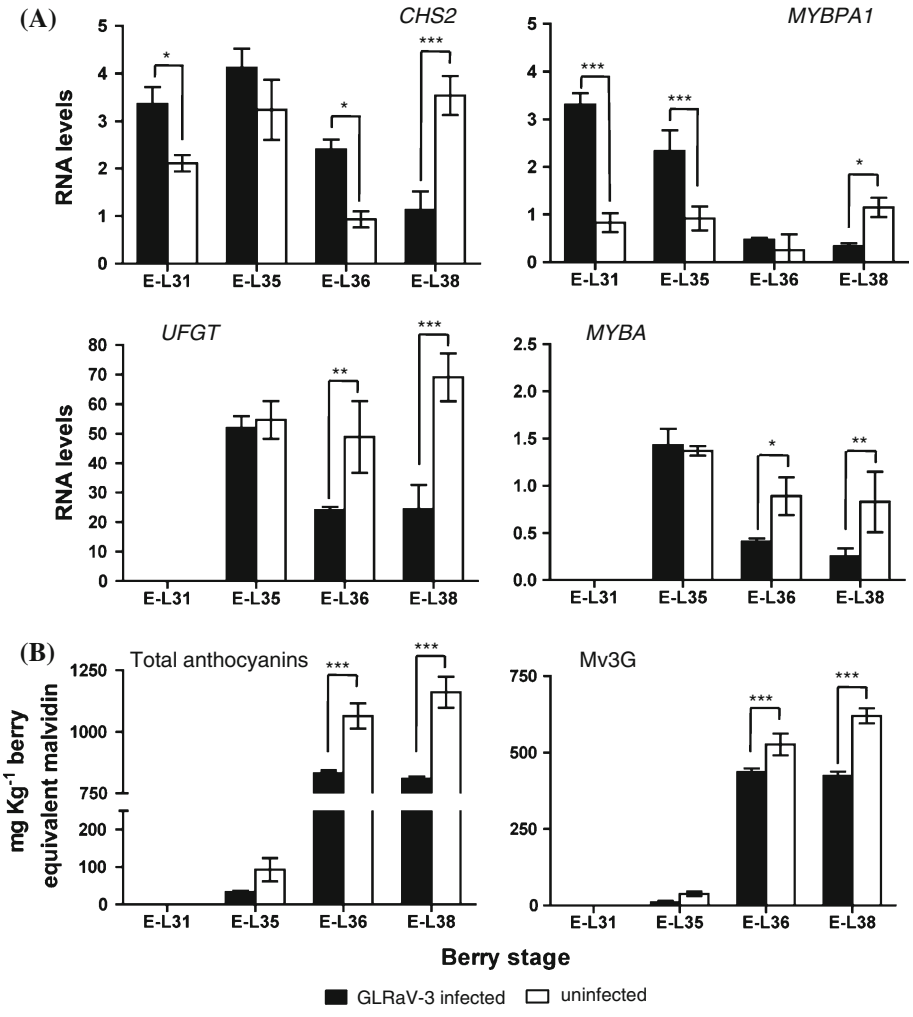


Fig. 5 Changes in mRNA levels of genes encoding for the anthocyanin biosynthesis pathway and anthocyanin concentration in response to the virus infection at four berry developmental stages. **a** The figure shows transcript abundance of anthocyanin biosynthetic genes (*CHS2* and *UFGT*) and the MYBs-associated transcription factors genes (*MYBA* and *MYBPA1*) in uninfected (*white bar*) and virus-infected grapevines (*black bar*). **b** The figure shows the total concentration of anthocyanin compounds and malvidin-3-O-glucoside (Mv3G) expressed as mg kg^{-1} of berries of malvidin equivalents at three ripening stages in uninfected (*white bar*) and virus-infected grapevines (*black bar*). Veraison (E-L35) corresponds to 8 WAF. Each graph represents the mean ± standard deviation of three biological replicates. Statistical significance is marked by *one asterisk* ($P < 0.05$), *two asterisk* ($P < 0.01$) and *three asterisk* ($P < 0.001$)

pattern observed for total anthocyanin levels (Fig. 5b). Other measured anthocyanins include delphinidin-3-O-glucoside (Dp3G), cyanidin-3-O-glucoside (Cy3G), petunidin-3-O-glucoside (Pt3G) and peonidin-3-O-glucoside (Po3G) (Table S8).

We also observed changes in the transcript levels for representative genes of the flavonol biosynthetic pathway in infected berries. As shown in Fig. 6a, the mRNA levels of *FLS1* were significantly lower at ripening (E-L38) in virus-infected berries. Accordingly, we observed changes in the flavonols content in infected berries during fruit development as compared with uninfected berries. As shown in Fig. 6b, these organic compounds increased in infected berries as compared with uninfected berries in E-L31 and E-L35 developmental stages. However, after veraison (E-L35) the decrease in flavonol concentration was more pronounced in virus-infected berries as compared to uninfected plants (Fig. 6b). A schematic of the results summarizing the gene regulatory networks associated with anthocyanin biosynthesis and sugar accumulation in response to virus infection is shown (Fig. 7).

Discussion

The relationship between viral infections and delayed ripening in grapevines was postulated long time ago (Martelli 1993). Nevertheless, support for this hypothesis and the characterization of the underlying molecular changes have not been reported. As shown here, the systemic viral infection by GLRaV-3 in Cabernet Sauvignon vines alters the gene expression profile in berries during ripening, interrupting the normal berry maturation process observed in non-infected vines. Importantly, the global gene expression pattern changes produced by viral infection translate into changes in the accumulation of different metabolites that explain characteristic phenotypes of infected berries.

Berry ripening is a dynamic process that involves global regulation of gene expression (Coombe and McCarthy 2000; Terrier et al. 2005; Deluc et al. 2007; Zenoni et al. 2010) and changes in the accumulation of sugar and secondary metabolites (Coombe 1992; Boss et al. 1996). Our results showed that several genes associated with the biological process "biosynthesis of primary and secondary metabolites" were repressed in infected berries, indicating an alteration of fruit metabolic processes due to the GLRaV-3 infection. For instance, we found altered expression of genes involved in sugar transport, hormone response and anthocyanin biosynthesis pathway during ripening due to the virus infection. Not surprisingly, these processes are key factors that affect fruit ripening and quality (Terrier et al. 2005; Deluc et al. 2007, 2009).

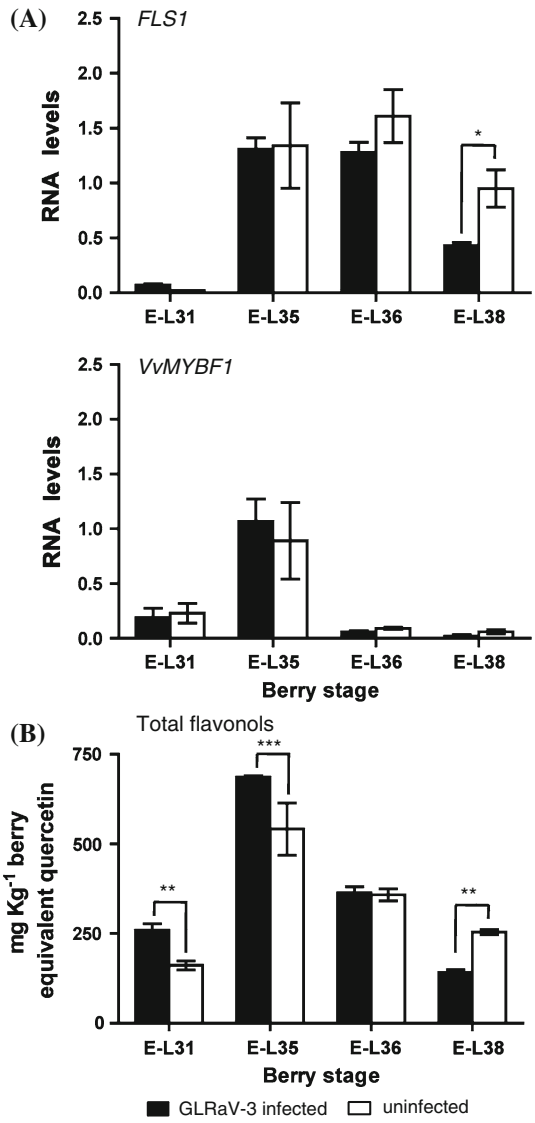
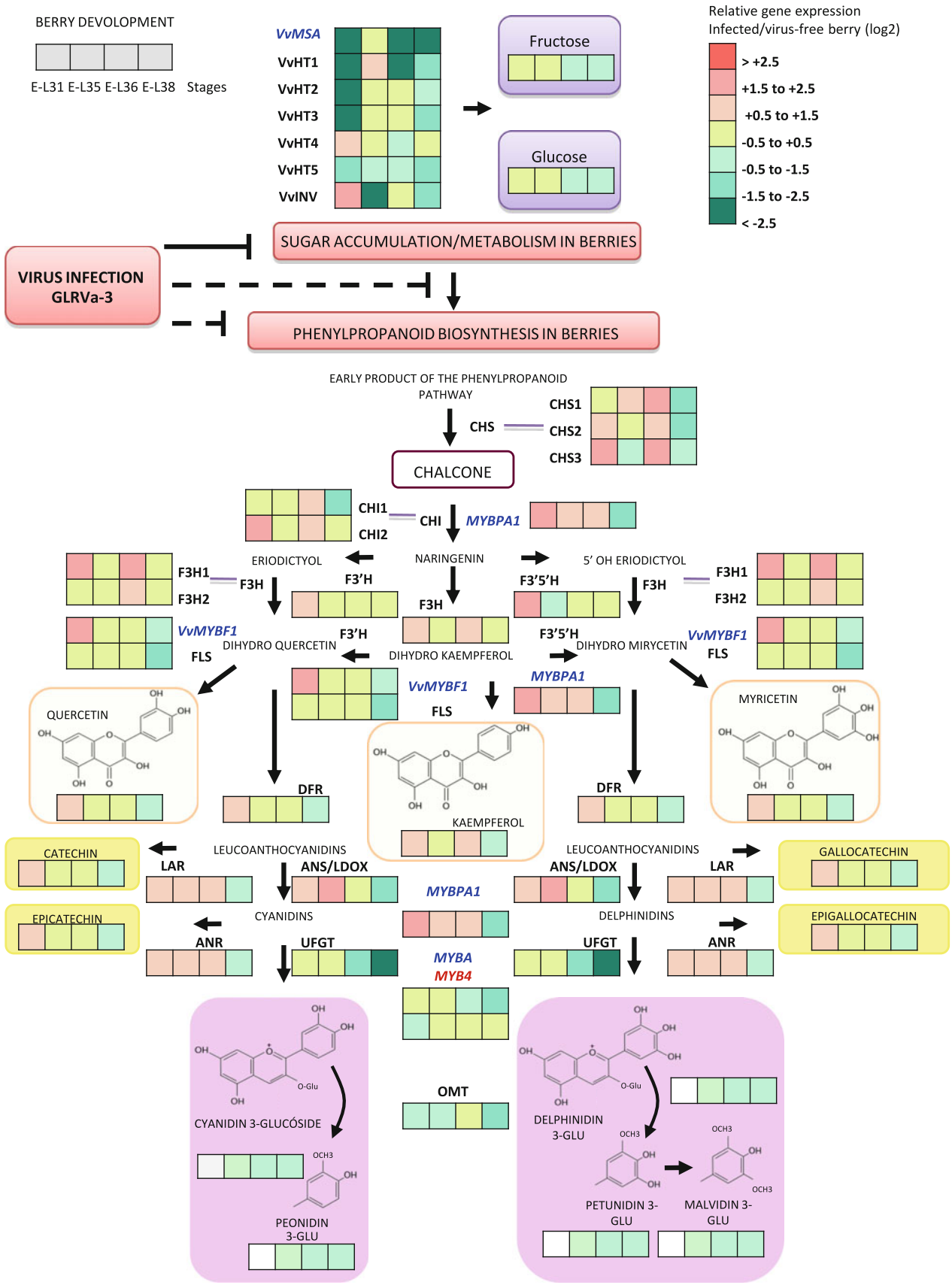


Fig. 6 Changes in mRNA levels of genes encoding for the flavonol biosynthesis pathway and total flavonol concentration in response to GLRaV-3 at four berry developmental stages. The figure shows transcript abundance of the **a** flavonol biosynthetic genes *FLS1* and its transcriptional regulator *VvMYBF1* and **b** total concentration of flavonol compounds at four ripening stages in uninfected (*white bar*) and virus-infected (*black bar*) grapevines. Veraison (E-L35) corresponds to 8 WAF. Each graph represents the mean ± standard deviation of three biological replicates. Statistical significance is marked by *one asterisk* ($P < 0.05$), *two asterisk* ($P < 0.01$) and *three asterisk* ($P < 0.001$)

We showed that GLRaV-3 repressed sugar concentration during ripening, possible due to alterations in the expression of sugar transporter genes and transcript involved in sugar metabolism. It has been reported that sugar transporters contribute to sugar accumulation during berry ripening (Vignault et al. 2005; Conde et al. 2006; Hayes et al. 2007; Afoufa-Bastien et al. 2010). In addition, viruses localized to phloem cells (Lartey et al. 1997) alter


BERRY DEVOLOPMENT
E-L31 E-L35 E-L36 E-L38 Stages
VvMSA
VvHT1
VvHT2
VvHT3
VvHT4
VvHT5
VvINV
Fructose
Glucose
Relative gene expression
Infected/virus-free berry (log2)
> +2.5
+1.5 to +2.5
+0.5 to +1.5
-0.5 to +0.5
-0.5 to -1.5
-1.5 to -2.5
< -2.5
SUGAR ACCUMULATION/METABOLISM IN BERRIES
VIRUS INFECTION
GLRVa-3
PHENYLPROPANOID BIOSYNTHESIS IN BERRIES
EARLY PRODUCT OF THE PHENYLPROPANOID PATHWAY
CHS
CHS1
CHS2
CHS3
CHALCONE
CHI1
CHI2
CHI
MYBPA1
ERIODICTYOL
NARINGENIN
5' OH ERIODICTYOL
F3H1
F3H2
F3H
F3'H
F3'5'H
VvMYBF1
FLS
DIHYDRO QUERCETIN
DIHYDRO KAEMPFEROL
DIHYDRO MIRYCETIN
QUERCETIN
KAEMPFEROL
MYRICETIN
DFR
CATECHIN
EPICATECHIN
GALLOCATECHIN
EPIGALLOCATECHIN
LEUCOANTHOCYANIDINS
LAR
ANS/LDOX
ANR
UFGT
CYANIDINS
DELPHINIDINS
MYBA
MYB4
OMT
CYANIDIN 3-GLUCÓSIDE
PEONIDIN 3-GLU
DELPHINIDIN 3-GLU
PETUNIDIN 3-GLU
MALVIDIN 3-GLU
O-Glu
OCH3

◀ **Fig. 7** Overview of the effects of the virus on transcripts and metabolites associated with sugar and phenylpropanoid pathways during berry ripening. These pathway were drawn based on described information (Hümmer and Schreier 2008; Hayes et al. 2007; Conde et al. 2006; Boss et al.1996). Transcript profile of genes that encoded five hexose transporters (*VvHT1-5*), a transcription factor (*VvMSA*) and an invertase vacuolar encoding gene (*VvINV*) are represented in response to virus during ripening. The phenylpropanoid pathway and some MYB transcription factors that regulate anthocyanin and flavonol biosynthesis genes in grape are also shown. Transcripts encoding for all transporters, enzymes and their corresponding transcriptional regulators (MYB-encoding genes and MSA-encoding gene) represented in this figure were analyzed in this work by RT-qPCR. The transcriptional repressor MYB4 is shown in *red*, while MYB and MSA activators are shown in *blue*. Relative transcript abundance are indicated in *green* if transcript levels declined, and in orange if mRNA levels increased compared with uninfected berries at the same developmental stage. The relative expression levels are represented using a *colored-scale* legend in the *upper right corner* of the figure. Metabolites (*filled squares*) were analyzed by HPLC and they are indicated in *green* if metabolite concentration declined or in *orange* if metabolite concentration increased in virus-infected vines in comparison with its control non-infected stage. *HT* hexose transporter, *INV* invertase vacuolar, *CHS* chalcone synthase, *CHI* chalcone isomerase, *F3H/F3'H/F3'5'H* flavonoid hydroxylases, *FLS* flavonol synthase, *DFR* dihydroflavonol reductase, *ANS/LDOX* anthocyanidin synthase/leucoanthocyanidin dioxygenase, *LAR* leucoanthocyanidin reductase, *ANR* anthocyanidin reductase, *UFGT* UDP-glucose flavonoid 3-O-glucosyltransferase, *OMT O*-methyl-transferase

the source-sink relationship and transport in *Nicotiana benthamiana* and *Cucumis melo* (Cheng et al. 2000; Shalitin and Wolf 2000; Gosalvez-Bernal et al. 2008). We have previously reported that sugar transporter genes are induced in leaves from grapevine infected by GLRaV-3 (Espinoza et al. 2007a). Induction of sugar transporter genes in photosynthetic organs in response to fungal infection has been correlated with sugar accumulation at the source in *Arabidopsis*, tomato and grape (Fotopoulos et al. 2003; Garcia-Rodriguez and Azcon-Aguilar 2005; Hayes et al. 2010). Additionally, it have been described a negative effects in photosynthesis (Christov et al. 2007) and accumulation of anthocyanins metabolites (Gutha et al. 2010) in leaves from GLRaV-3 infected vines. Together, these results could explain the reduced sugar levels measured in the GLRaV-3 infected fruits under our experimental conditions and the reduction in sugar levels measured in the must of GLRaV-3-infected grapevines (Borgo and Angelini, 2002). More interestingly, sugars can regulate global gene expression in *Arabidopsis* including the anthocyanin biosynthesis pathway (Teng et al. 2005; Solfanelli et al. 2006). In *Vitis*, it is known that anthocyanin levels increase concomitantly with sugar accumulation (Coombe, 1992; Vitrac et al. 2000; Deluc et al. 2009). The repression of genes involved in the anthocyanin pathway by the virus infection observed in this study is probably due to the lower sugar content in the infected berries throughout fruit development, as shown in Fig. 7. Our results are consistent with a model where the virus presence in the vasculature alters source/sink balance and sugar transport affecting sugar levels. In addition to this model, also sugar metabolism and photosynthesis could be affected by the virus. Consistent with both ideas, grapevine anthocyanin biosynthetic genes such as *LDOX* and *DFR* have regulatory elements know as "sucrose boxes" in their promoter regions (Gollop et al. 2001, 2002), which are required for sugar-specific responsiveness (Atanassova et al. 2003). In *Arabidopsis*, this process is regulated by a MYB transcription factor (Teng et al. 2005). Our results suggest the connection between the aforementioned *cis*-acting element and the MYB transcription factor is conserved in grapes.

In addition, our results were consistently with the delayed ripening commonly observed in virus infected berries (Martelli 1993; Borgo and Angelini 2002). Accordingly, we showed that virus infection triggers an incomplete ripening transcriptome and many genes normally induced during ripening in uninfected plants were not induced in GLRaV-3 infected fruits. Among these genes, approximately 10% do not possess a known biological function, representing therefore an interesting group of genes for further investigation.

Anthocyanin metabolites are important quality compounds for grapes and have important physiological functions, for example as protection molecules under stress conditions (Boss et al. 1996; Deluc et al. 2007; Pilati et al. 2007; Lund et al. 2008; Deluc et al. 2009). We showed that total anthocyanin accumulation was significantly lower in GLRaV-3 infected berries during ripening. Notably, the expression of the *UFGT* and its transcription factor MYBA, key genes in anthocyanin biosynthesis (Kobayashi et al. 2002, 2004; Walker et al. 2007), were strongly repressed due to the virus infection. It has been described that diverse abiotic stresses are also capable to modify flavonoid-related genes, and their synthesis in grapevine (Yamane et al. 2006; Mori et al. 2007; Grimplet et al. 2007; Deluc et al. 2009) and other plants (Christie et al. 1994; Lo Piero et al. 2005). For instance, water deficit and sunlight exposure could affect the berry metabolism and accelerate the anthocyanin biosynthesis (Castellarin et al. 2007; Deluc et al. 2009; Matus et al. 2009). We observed that the virus infection also affected the berry metabolism and repressed anthocyanin biosynthesis. This finding suggests an effect in the phenylpropanoid pathway that seems not to be exclusively related to abiotic stresses. Supporting this idea, it has been described that phenylpropanoid genes are also affected by a fungal compatible infection (Rotter et al. 2009).

Although GLRaV-3 causes a systemic infection in vines, genes described in plant defense mechanisms were induced in infected-berries at E-L38 (Table 1), an observation that correlates with an increment in the GLRaV-3 RNA levels during ripening. These findings suggest that the virus triggers a defense-like plant response within the berry. This

response could also explain in part the changes in berry metabolite profiles resulting in the altered sugar reallocation within the infected-berries. For example, we observed that a *Patatin-like* gene was induced. In *Arabidopsis*, this gene contributes to the resistance to Cucumber mosaic virus, inducing a hypersensitive response (HR) in the plant (La Camera et al. 2008). In addition, a transcript encoding a putative DICER-LIKE protein, enzyme implicated in the virus-induced gene silencing (Bernstein et al. 2001; Blevins et al. 2006), as well as proteins involved in plant defense response to viruses (LRR and SIZ1, Ascencio-Ibáñez et al. 2008) and fungal pathogens (LOX2, Bell and Mullet 1993; Jensen et al. 2002) also showed an increment in their respective mRNA levels in infected-berries. Nevertheless, the plant was unable to stop the viral infection. Accordingly, a putative cyclin-dependent protein kinase (CYCT1;4) and SOBER1-like gene were up-regulated in infected-berries. These genes play an important role in virus infections (Cui et al. 2007) and in the suppression of the HR (Kirik and Mudgett 2009), respectively.

Our data provide important insights into the changes associated to development during compatible virus infection in fruits. This work contributes to understanding of the molecular mechanisms behind the viruses-triggered changes on grapevine physiology and berry maturation, and provides putative biotechnological targets for improving fruit quality in virus-infected grapevines.

Acknowledgments This work was supported by CORFO-Innova 07Genoma01, Millennium Nucleus for Plant Functional Genomics (P06-009-F) and FONDECYT 1100709. We are grateful to Dr. Michael Handford (Universidad de Chile) for critically reading the manuscript and assistance in language support. We also thank Hector Morales for his contribution in HPLC-DAD analysis.

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