IMPACT OF GRAPEVINE LEAFROLL VIRUS INFECTIONS ON VINE PHYSIOLOGY AND THE BERRY TRANSCRIPTOME

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Abstract:
Context and purpose of the study - Grapevine leafroll associated virus (GLRaV) infections deteriorate vine physiological performance and cause high losses of yield and fruit quality and are thus causing serious economic losses in the wine industry. Physiological and molecular studies on the impact of leafroll infections on plant and fruit metabolism are relatively scare and the molecular mechanisms associated with the plant response to the virus during berry ripening are not well understood so far. Commonly observed phenotypic alterations consist in a ripening delay, a reduction in anthocyanin and sugar accumulation. The few molecular studies associated in particular the lack of anthocyanin of berries of infected vines to a repression of key genes of anthocyanin biosynthesis. However such studies did never account for berry heterogeneity and potential phenological shifts induced by virus infection, which could have introduced biases in gene expression studies.
Material and methods - In the present study a long-term experiment was established in the year 2000, with the aim to investigate the effects of infections with different GLRaVs (GLRaV 1 & GLRaV 1+3) on vine and grape physiology. Physiological data (yield, vigor, photosynthesis, berry quality) has been collected from 2015 to 2018. In 2018 a transcriptomic (RNA-seq) analysis of 2 reconstituted berry ripening stages was performed. Therefore 245 berries were individually sampled and individually analyzed for sugar and organic acids in order to re-constitute 2 homogenous ripening stages to circumvent intercluster berry heterogeneity and thus to compensate for phenological shifts induced by virus infections. RNA of reconstituted samples was extracted and sequenced by single end sequencing and subsequently analyzed for differentially expressed genes (DEGs).
Results - Physiological measurement showed a significant decrease in photosynthesis, yield and sugar content, which were highly significant in the co-infected vines (GLRaV 1+3).
RNA-sequencing of berries revealed a total of 2136 DEGs between control and virus infections. Several transcriptions factors related to abiotic and biotic stress could be identified and showed interesting variation in dependence to ripening stage and infection severity. Strikingly previously reported repression of the anthocyanin biosynthesis and sugar metabolism could not be confirmed by gene expression. This illustrates that the main damaging effect on GLRaV infection is rather related to a phenological shift than to a direct impact on metabolism. The here reported results give new insight in the mechanism of leafroll infection and emphasize the importance of the sampling protocol of molecular studies investigating berry metabolism.

Keywords: Grapevine leafroll virus, GLRaV 1, GLRaV 3, RNA-seq, berry metabolism.

1. Introduction

Grapevine (*Vitis vinifera* L.) is amongst the most important perennial fruit crops (OIV, 2018) and has been propagated vegetatively and distributed worldwide for thousands of years. This is the main reason why it is the host of a very important number (currently >70) of graft- and vector transmitted viral diseases that cause important economic losses in all wine growing regions (Maliogka et al., 2015). Grapevine leafroll disease (GLD) is considered to be the most widespread and devastating virus associated disease. So far five distinct viral species from the *Closteroviridae* family were associated with GLD, named grapevine leafroll associated virus(GLRaV-1 to 4 and 7) , with the most widespread ones being GLRaV-1 and GLRaV-3 (Velasco et al., 2014, Peake et al., 2004, Maree et al., 2013). Several studies showed that GLD cause important reductions in yield, vigor and longevity of vines, that it delays fruit

ripening, impedes fruit pigmentation and reduces sugar accumulation (Naidu et al., 2014). However, the physiological mechanisms that are triggered by the virus in the host plant and that underly its detrimental effects on fruit quality are far from being understood. The scarce molecular studies on ripening berries that assessed the impact of GLD infection on berry metabolism showed that key genes within the anthocyanin biosynthetic pathways and sugar metabolism are repressed by GLD (Vega et al., 2011). Such studies however did not account for the intra-cluster berry heterogeneity of ripening grapes (Gouthu et al., 2014) which can introduce important biases in molecular studies and mask transcriptomic effects (Rienth et al., 2016, Rienth et al., 2014b, Carbonell-Bejerano et al., 2016).
The present study aimed therefore to characterize the physiological and transcriptomic effect of GLD infections on vine and on berry by accounting for intra-cluster berry heterogeneity.

2. Material and methods

Treatments consisted of 3 times 10 vines (cultivar Pinot noir) that were infected by grafting (with 5BB rootstock) with either GLRaV 1 (Treatment1; T1) or co-infected with GLRaV 1 plus GLRaV 3 (Treatment2; T2) or not infected (control)
Vine physiology was characterized by measurements of photosynthesis (Ciras 3, ppsytems®), vigor (pruning weight), yield, sugar and organic acid analysis by FTIR and HPLC.
For transcriptomic analysis berry samples were drawn at two different ripening stages. The first stage (S1) was sampled at véraison (14.08.2017), when ca 50% of berries were colored, berries from 5 vines per treatment were randomly chosen. The second sampling (S2) was performed 15 days later (29.08.2017) at mid-ripening on the 5 other vines, that have not been sampled during S1. For each sampling stage berries were individually wrapped in aluminum and frozen in N_2. Each berry was individually crushed under N_2 and an aliquot of powder was subsequently taken, diluted and analyzed for sugar and organic acids by HPLC. According to sugar concentration and the malic to tartaric acid ratio of individual berries, 6 batches of each sampling date and treatment were re-constituted for RNA-extraction, resulting in 18 total samples to be extracted including replicates. RNA was extracted according to Rienth et al. (2014a), RNA quality was assessed by an Agilent 2100 Bioanalyzer (Agilent Technolgies). TruSeq Stranded mRNA-seq (Illumina, San Diego, CA) was used for library preparation and sequenced on 75 bp single-end mode with a NextSeq500 apparatus (Illumina). Gene annotation was derived from Grimplet et al. (2012)
Differentially expressed genes (DEG) were identified using the R package DESeq on count data. Pairwise comparison was performed between uninfected and virus-infected (GLRaV 1 and GLRaV 1+3) conditions at two sampling stages separately and between transcripts were considered as significantly modulated or differentially expressed with the following criteria: the absolute fold change was > 2 (log2 fold change < -1; > +1) and the FDR-adjusted p-value < 0.05. Principal component analysis (PCA) was performed using R on normalized count data (Figure 2). K-means clustering was performed using MeV on normalized mean centered logs of DEGs (Figure 3). Functional enrichment of deregulated genes as well as in genes clusters was performed using FatiGO (Al-Shahrour et al., 2004)

3. Results and discussion

Physiological performance of GLRaV infected vines confirms results reported in the literature where GLD had a general negative impact on overall vine physiology and berry quality (Alabi et al., 2016). The co-infection (T2) with GLRaV1+3 had a much more detrimental impact on physiological performance of vines. This resulted in a reduction of vigor, assessed by pruning weight, which decreased by around 15 % in GLRaV1 infected vines (T1) and up to 80 % in GLRaV1+3 (T2) vines in 2015, 2016 and 2017 (data not shown). Sugar concentration was not significantly different between control and treatments in 2015 but in 2016 and 2017, where it was reduced by 20% for T2 in comparison to controls. T1 reduced sugar concentration in berries only by 5%. Such a high variation of symptom expression between years could be owed to vintage variations as reported in previous GLD studies (Alabi et al., 2016). The rather low difference in sugar concentration between treatments as compared to differences in vigor can partly be attributed to a lower berry weight leading to a higher concentration of solutes in berries of infected vines, which indicates as well that infected vines accumulate lower absolute quantity of sugar on a per berry basis. This is congruent with an overall lower carbon status of infected vines as indicated by lower vigor but as well by photosynthesis which showed a reduction of in average 70% in T2 in all years (data not shown).

Figure 1 shows the malic to tartaric acid ratio (MA/TA) plotted against sugar concentration of individually analyzed berries. As expected, due to the respiration of malic acid post-véraison, which occurs simultaneously to sugar accumulation, the MA/TA ratio decreases throughout ripening when sugar concentration increase (Ollat et al., 2002), independently of changes in berry weight. More than two fold variation of sugar concentration in controls, ranging from 95 to 220 $g.L^{-1}$ (Figure 1) within a very narrow window of 15 days confirms the very high asynchrony of individual berry as already addressed in several studies (Shahood et al., 2015, Gouthu et al., 2014, Rienth et al., 2016). This heterogeneity becomes even more important when berries of virus infected vines are considered, which is probably owed to the delay in phenology mainly induced by T2 in S1. From Figure 1 it can be hypothesized that T2 had the greatest impact on berry physiology during stage 1 (S1), where it delayed berry ripening, or maybe the transition from the lag phase to véraison the most. This very high vulnerability of the grapevine berry to biotic and abiotic stresses around véraison has been highlighted in previous studies (Rienth et al., 2016, Rienth et al., 2014b). T2 in S1 seemed also to have limited berry heterogeneity during early ripening probably due to the delay in the onset of véraison.
The large heterogeneity visualized in Figure 1 highlights the necessity to reconstitute homogenous batched of individual berries according to their biochemical characteristics prior to RNA extraction. Without this reconstitution, DEG analysis would yield only in the observation of changes in ripening related transcripts due to the phenological delay induced by virus infection.
For RNA sequencing, individual berries from Controls, T1 and T2 were reconstituted with following characteristics, for stage one: sugar concentrations between 99 – 117 $g.L^{-1}$ and a MA/TA ratio between 0.74 to 0.98 and for stage 2: sugar concentration between 162 to 190 $g.L^{-1}$ with a MA/TA ranging between 0.38 to 0.57.

Analysis of DEGs between control and treatments at the two stages yielded in a total of 2136 deregulated transcripts as illustrated in table 1.
The early berry development stage just after the transition from the lag phase (herbaceous plateau) to véraison seemed again to be the most sensitive stage with highest virus induced transcriptomic changes even though batch reconstitution accounted for the delay in phenology. Interestingly the highest induced variation in gene expression was caused by T1. This is highlighted by absolute number of total DEGs (Table 1) but as well by PCA in Figure 2 and hierarchical clustering in Figure 3.

By k-means clustering of mean centered log expressions of all DEGs (Figure 3) it was possible to separate genes in 10 different clusters in a stage and treatment dependent manner. For example, genes allocated to cluster 1 (cl1), cluster (cl3) and cluster (cl4) were highly induced by coinfection (T2), whereas genes of cl1 were triggered in both stages by T2 and genes in cl4 only in S1. Enriched functional categories (FCs) within cl1 were mainly linked to xyloglucan and pectin modifications whereas predominant FCs in cl4 were related to ABA-mediated signaling, abiotic stress response and heat shock mediated protein folding. Whereas transcripts allocated to cl9 are highly repressed by T2 in both stages. Genes within cl9 are linked to starch catabolism, jasmonate-mediated signaling, Tyrosine catabolism and phenylpropanoid biosynthesis. Genes that where predominantly developmentally regulated and less by virus infection are allocated to cl2 and cl8 and comprise categories involved for example in flavonoid synthesis and photosynthesis.
As regards the downregulation of phenylpropanoid synthesis which has been reported previously in several studies (Vega et al., 2011), here, only a partial repression of early phenylpropanoid synthesis could be detected as seen in FCs within genes allocated to cl9. Individual genes and key transcription factors within this pathway, notably proanthocyanidin synthesis regulated by key-enzymes such as *phenylalanine lyase (PAL), chalcone synthase (CHS)* and the main regulator, the transcription factor *MYBPA1* (Bogs et al., 2007) were congruently downregulated by T1 and more by T2 only during stage 1. Strikingly and in contradiction to previous studies (Vega et al., 2011) further downstream of proanthocyanidins, key genes of the anthocyanin biosynthesis, pathway such as *MYBA1* or *UFGT* (Azuma, 2018, Boss et al., 1996) were not repressed by GLD which highlights that previously observed effects on anthocyanin biosynthesis by GLD infection are mainly due to phenological delay of berries that were sampled at the same time point and not the same ripening stage, which is also confirmed by hexoses transporter (HT1-HT6) which were not repressed by GLD as opposed to previous studies (Vega et al., 2011)

4. Conclusions

To our knowledge this is the first comprehensive molecular study conducted on GLD graft infected grapevines comparing different infections (GLRaV 1 and GLRaV 1+3) in an experimental plot under relatively homogenous environmental conditions in comparison to previous field studies. The strict berry selection according biochemical characteristics enabled us to account for potentially induced phenological shift by GLD and to decipher a phenology independent transcriptomic response of the berry to GLD. By this way it was possible to illustrate that GLD does not cause an active inhibition of the anthocyanin pathway but that this often in the literature reported consequence is mainly due to the phenological shift induced by leafroll virus infections.

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Table 1. Number of different DEGs in C, T1 and T2 samples on the two stages of sampling S1 and S2

	Total DEGs	Up Regulated DEGs	Down Regulated DEGs
S1_C_T1	859	541	318
S1_C_T2	741	392	349
S1_T1_T2	1078	501	577
S2_C_T1	250	12	238
S2_C_T2	503	249	254
S2_T1_T2	502	373	129

DEGs: Differentially Expressed Genes

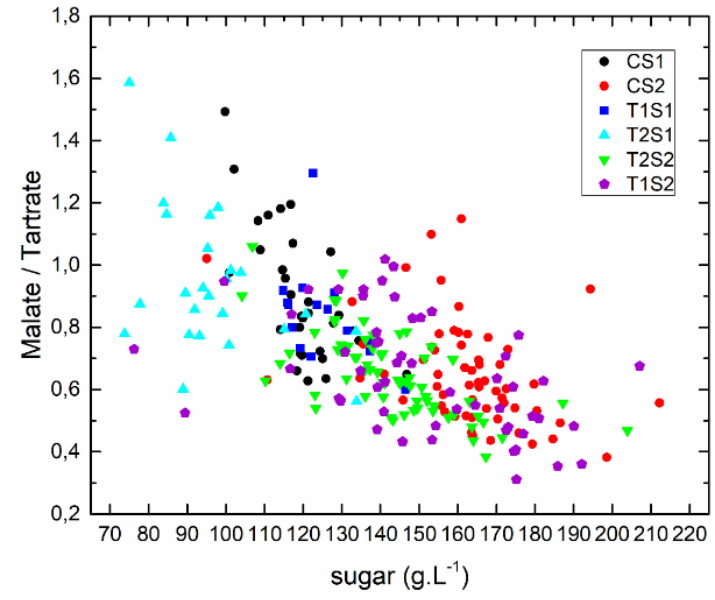

Figure 1: Biochemical characteristics of all individually analysed berries for all treatments and stages. **CS1:** Control Stage 1; **CS2:** Control Stage 2; **T1S1:** Treatment GLRaV 1 Stage 1; **T1S2:** Treatment GLRaV 1 Stage 2; **T2S1:** Treatment GLRaV 1+3 Stage 1; **T2S2:** Treatment GLRaV 1+3 Stage 2

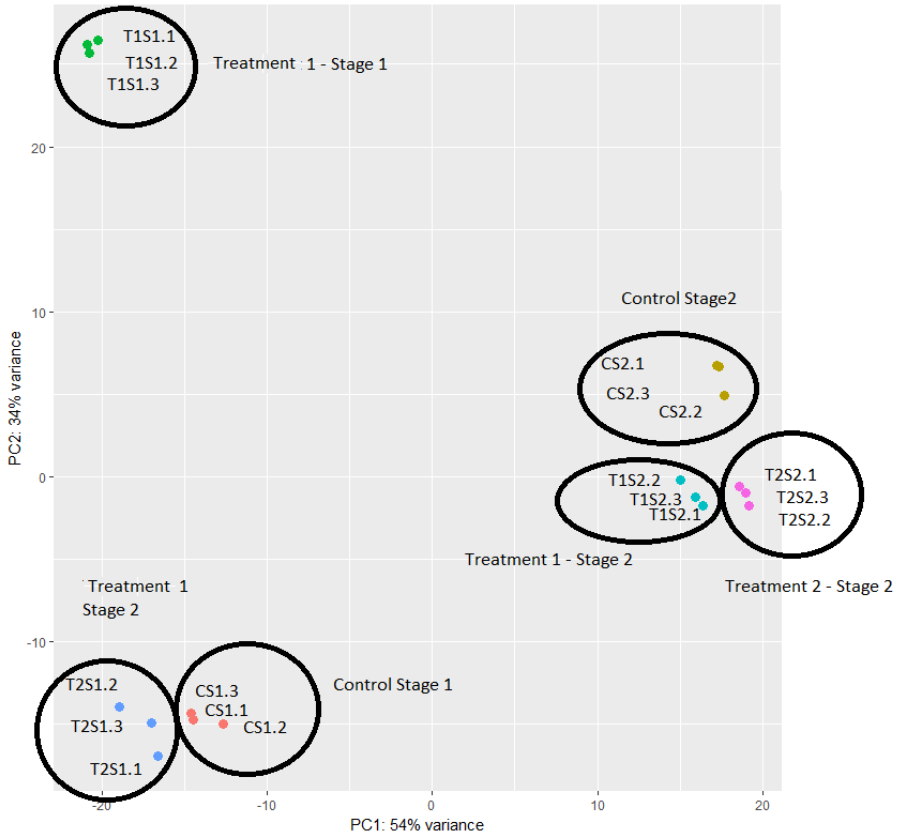

Figure 2: Principal component analysis on normalized count data for all treatments and stages with triplicates. **CS1:** Control Stage 1; **CS2:** Control Stage 2; **T1S1:** Treatment GLRaV 1 Stage 1; **T1S2:** Treatment GLRaV 1 Stage 2; **T2S1:** Treatment GLRaV 1+3 Stage 1; **T2S2:** Treatment GLRaV 1+3 Stage 2.

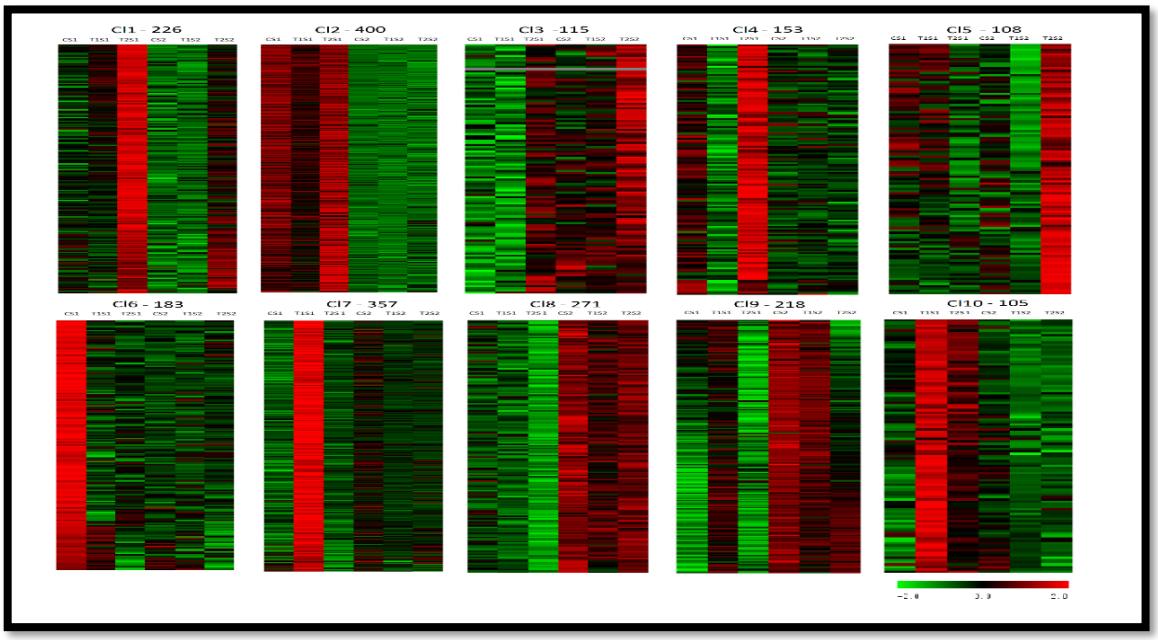

Figure 3: K-means clustering on DEG on log transformed and mean-centered expressions. **CS1:** Control Stage 1; **CS2:** Control Stage 2; **T1S1:** Treatment GLRaV 1 Stage 1; **T1S2:** Treatment GLRaV 1 Stage 2; **T2S1:** Treatment GLRaV 1+3 Stage 1; **T2S2:** Treatment GLRaV 1+3 Stage 2.

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