

Research Paper

Unraveling the key molecular events of Pinot noir berry ripening under varying crop load

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ABSTRACT

Aligned to exploring the physiological and molecular complexity of grape berry development, there is a need to characterize the influence of the source:sink relationships on the genetic regulation of fruit composition. Crop load, as defined by the amount of fruit produced per unit vegetative growth at dormancy, is a common measure of source:sink relationships used to evaluate vineyard production efficiency. We studied the impact of varying crop load on the transcriptome and metabolome of Pinot noir grape berries by comparing the development and ripening of fruit grown on vines with either 50 % or 75 % of their grape clusters removed immediately following fruit set compared to unthinned vines for three consecutive vintages. A clear impact on the general phenylpropanoid pathway resulting in a redistribution between stilbenes and anthocyanins was revealed under varying crop loads and consistent with the transcriptomic profiles of the corresponding branches. Moreover, we identified genes, such as *LBD1a3* and *AG2*, modulated by crop load around veraison, representing putative transcriptional key triggers of the berry ripening phase responding to differences in the vine source:sink ratio generated by the application of cluster thinning. Genes, specifically *EXPA1* and *EXPA18*, involved in softening and other crucial events of ripening initiation responded to crop load and likely influenced the progression of the ripening process. Beyond the major impacts represented by a shift of the onset and completion of ripening, we were able to highlight more subtle effects of the crop load, related to the rate at which the molecular and metabolic changes occur. This study asserts that grape metabolism and transcriptome are remarkably flexible, and that manipulations such as cluster thinning induce extensive, genome-wide changes in expression during berry development. The insights gained here pave the way to progress towards the construction of robust models depicting the molecular network that characterizes berry development and the impact of crop load on its molecular regulation.

1. Introduction

The economic relevance of the grapevine (*Vitis vinifera* L.) berries and their processed products has driven extensive research efforts to characterize the physiological and molecular complexity of grape berry development (Fasoli et al., 2018; Tornielli et al., 2023). This can be framed into the goal of elucidating how pathways controlling the formation of different quality traits are interlinked at the berry level and how they relate to the final physical–chemical and sensory composition

of wine (Previtali et al., 2022).

Berry development follows a double sigmoidal curve with two phases of growth separated by a lag phase (Coombe, 1992; Dokoozlian, 2000). The first growth phase is characterized by pericarp enlargement caused by cell division and elongation, during which the berries accumulate organic acids, tannins and other phenolic compounds but little sugar, remaining green and hard. Berry growth slows during lag phase, marked by the transition to ripening (veraison) when the seeds are mature. The second growth phase involves further changes that make the fruit edible

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and attractive promoting seed dispersal, including change in skin color, water influx, berry softening, accumulation of sugars, loss of organic acids and tannins, and synthesis of volatile aromas (Conde et al., 2007). Hormones play a central role during the berry developmental program, participating in the regulation of the different phases. In particular, auxin indole-3-acetic acid (IAA) acts as inhibitor of ripening, whereas abscisic acid (ABA) induces it (Cawthon and Morris, 1982; Böttcher et al., 2010, 2013). Ethylene is also involved in ripening stimulation, however its role in the ripening of non-climacteric fruits, such as grapevine berries, is less clear than in climacteric fruit maturation (Vendrell and Palomer, 1998; Davies and Böttcher, 2009).

The general phenylpropanoid pathway leading to the production of berry phenolic compounds contributing to color, mouthfeel and wine stability (e.g., anthocyanins, tannins and stilbenes) is well elucidated (Kobayashi et al., 2001; Cavallini et al., 2015; Davies et al., 2020). Phenylpropanoids originate from the amino acid phenylalanine through the action of PHENYLALANINE AMMONIA LYASE (PAL), controlling the first committed enzymatic step of the pathway. The subsequent steps controlled by CINNAMATE 4-HYDROXYLASE (C4H) and 4-COUMARATE:COA LIGASE (4CL) lead to the production of p-coumaroyl-CoA representing a common substrate of several specialized branches. In this regard, CHALCONE SYNTHASE (CHS) uses p-coumaroyl-CoA for the biosynthesis of the flavonoids, like anthocyanins, through a multistep route and the final key of UDP GLUCOSE:FLAVONOID 3-O-GLUCOSYLTRANSFERASE (UGT; Kobayashi et al., 2001). In stilbene-synthesizing plant species like the grapevine the same substrate is used by STILBENE SYNTHASE (STS) for the biosynthesis of resveratrol and its derivatives (Schoppner and Kindl, 1984; Lanz et al., 1991). The phenylpropanoid pathway is strictly regulated at the transcriptional level during berry formation and ripening, by genetic, developmental, and environmental cues.

The availability of high-throughput analytical methods and a high-quality draft of the grapevine genome sequence (Jaillon et al., 2007) has resulted in the characterization of berry development at the transcriptomic and metabolomic levels (Guillaumie et al., 2011; Lijavetzky et al., 2012; Dal Santo et al., 2013; Palumbo et al., 2014; Anesi et al., 2015; Ghan et al., 2015; Savoi et al., 2016; Wong et al., 2016; Zenoni et al., 2016; Fasoli et al., 2018). Both these approaches can help dissecting the contribution of the agronomical practices on the final phenolic profile in ripe berries of field-grown grapevines.

With this regard, the manipulation of “crop load” represents an agronomic practice to influence fruit composition at multiple levels (Martinez-Luscher and Kurtural, 2023). Crop load estimates the source:sink relationship by measuring the ratio between the sink or crop size (i.e. yield per vine or per unit of land area) and the source or vine canopy size (assessed as pruning weight or leaf area). Generally, in grapevine a leaf area (source) of 10–15 cm² is required to fully ripen 1 g of fruit (sink), and this normally results in a yield:pruning weight ratio ranging between 5 and 10 (Kliewer and Dokoozlian, 2005). If crop load is lower than 5, the vine is considered to be sink limited or undercropped, and tends to partition comparatively more assimilates into vegetative growth rather than to fruit maturation. Conversely, if the crop load is greater than 10 the vine has insufficient leaf area to fully support fruit ripening and experiences source limitation (overcropping). When the relationship between crop size and vegetative growth is optimum with a yield:pruning weight ratio between 5 and 10, the vines are considered to be in balance and the potential for producing the highest quality fruit is achieved.

Crop load is typically manipulated by cluster thinning – the removal of grape cluster from the vine (Kliewer and Dokoozlian, 2005; Dokoozlian and Wolpert, 2009; VanderWeide et al., 2024). However, the impact of cluster thinning on berry ripening (i.e. technological maturity parameters such as sugar accumulation) and on the quality traits related to secondary metabolism (i.e. color, aroma and mouthfeel) is rather complicated and vary as a function of the cultivation region, seasonal conditions, cultivar, training system and thinning period

(Keller et al., 2005; Nuzzo and Matthews, 2006; Guidoni et al., 2008; Keller et al., 2008; Wang et al., 2018; Previtali et al., 2021; Skrab et al., 2021). To significantly affect the onset and rate of sugar accumulation, crop thinning should be done early in fruit development. At this stage, the fruit and leaves are competing for sugars and other nutrients (Parker et al., 2015). This carbon limitation can strongly impact fruit composition (Bobeica et al., 2015). Thinning clusters can prevent this carbon limitation and improve anthocyanins content and/or composition in the grapes (Dokoozlian and Hirschfeld, 1995; Palliotti and Cartechini, 2000; Guidoni et al., 2002, 2008; Wang et al., 2018; Sivilotti et al., 2020). Moreover, Pastore et al. (2011) reported an extensive transcriptome remodeling in grapes from Sangiovese vines thinned at veraison. The remodeling included carbohydrate metabolism, and synthesis and transport of secondary products. Genes regulating monoterpene metabolism were also revealed as differentially expressed in white varieties following thinning: the expression of four terpene synthase genes in Gewürztraminer grapes was promoted by early thinning, whereas the expression levels of genes involved in the terpenoid backbone biosynthetic pathway, such as three *DXS* genes, resulted modulated by the crop load in Muscat Hamburg grapes (Yue et al., 2021; Kovalenko et al., 2022). Further, modulation of specific genes of the phenylpropanoid pathway along with modification in anthocyanin composition were reported in Summer Black grapes due to cluster thinning (Xi et al., 2016). To understand better the molecular and metabolic responses of grapevines to cluster thinning we studied the impact of three crop load levels on the transcriptome and metabolome of Pinot noir grapes during development and ripening. Cluster removal was applied immediately after fruit set for three consecutive vintages. Weekly grape sample analyses by ultra-high-performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UHPLC-QTOF-MS) and RNA sequencing (RNA-Seq) were performed to reveal changes by crop load in the content of phenolic compounds during berry development and the corresponding transcriptomic profiles. This extensive sampling design also allowed identifying an early effect of crop load manipulation (i.e., around veraison) on genes that represent putative triggers of the ripening process (Fasoli et al., 2018). By investigating the key molecular events governing berry ripening when crop load is manipulated, this study aims to provide a foundational understanding that can enable viticulturists to better optimize source:sink ratios, thereby improving both fruit quality and overall production.

2. Materials and methods

2.1. Vineyard features

V. vinifera cultivar Pinot noir (clone FPS 23 grafted on Freedom rootstock and planted in 2001) was used in this study. Pinot noir vines were grown in a sandy clay loam soil and planted in an east–west row orientation with 3.0 m between rows and 1.5 m between vines. The vines were trained to quadrilateral cordons and trellised using a double cross-arm system. The vineyard was located eastern of Modesto (CA, USA; 37°39'02.8"N 120°50'03.6"W). This area features a warm climate with low topographical relief, similar to the characteristics of the Lodi and Madera AVAs of the California Central Valley (Jones et al., 2010).

2.2. Cluster thinning treatments

Cluster thinning treatments were applied immediately following fruit set as required to remove either 50 % (moderate thin) or 75 % (severe thin) of the clusters on each vine. Unthinned vines were not manipulated. The experimental vineyard was organized in a randomized complete block design. The treatments were applied on nine vineyard rows of approximately 150 vines: three row replicates per treatment. To allow weekly grape sample collection from post fruit set to maturity without impacting the crop load, 16 eight-vine blocks were designed along each vineyard row, namely for each replicate. At each time point,

one block of each replicate was randomly selected and sampled. The treatments were performed over three consecutive vintages (2012, 2013, 2014) in the same vineyard using nine different rows each year to exclude treatment effects carried over from previous growing seasons.

2.3. Harvest and pruning data

Once the grapes of each treatment reached commercial maturity for winemaking (total soluble solids (TSS) = 24.5 °Brix), the vines were harvested, and cluster number and total fruit weight was recorded (Table 1). TSS were determined using a PAL-1 Atago refractometer (Atago Inc., Bellevue, WA).

During dormancy, one-year old vegetative growth was removed from the vines, weighed, and recorded. Fruit and pruning weights were used to calculate the Ravaz Index (RI; Ravaz, 1903; Supplementary Table S1). Additional field data was collected and is reported in the Supplementary Dataset S1.

2.4. Grape data

2.4.1. Sampling strategy

Berries were collected at 10-day intervals in 2012, and weekly in 2013 and 2014, beginning at fruit-set and continuing to harvest. Berry phenology progression was monitored to define the veraison stage timing for both control and treated vines, as cluster thinning often determines an anticipation of veraison. Veraison was defined as the 50 % colored berries per cluster. Sample time points are presented by their distance to the recorded veraison stage (days after veraison, DAV) for each sampling series, namely, the control samples refer to the veraison date recorded for the control condition that year, likewise for the other conditions and vintages. All samples were collected at the same time of day (8:00 am). We collected 255 samples in total (Supplemental Dataset S2): 99 for control (30, 33 and 36 during vintages 2012, 2013 and 2014, respectively), 78 for the 50 % cluster thinning treatment (24, 27 and 27 during vintages 2012, 2013 and 2014, respectively) and 78 for the 75 % cluster thinning treatment (24, 27 and 27 during vintages 2012, 2013 and 2014, respectively). Each sample replicate comprised 26 clusters of berries from each vine block.

Table 1

Yield data by cluster thinning level. Yield data (kg/vine and tons/ha values) and fruit weight (berry and cluster weight measurements) were recorded when the grapes of each treatment reached commercial maturity for winemaking (total soluble solids (TSS) = 24.5 °Brix). Averaged values ($n = 3$) $\pm$ standard deviation are reported. Different letters to the right of the numbers denote significant differences by treatment within each vintage (Tukey's test, $p < 0.05$).

Treatment	Year	Yield (kg/ vine)		Yield (tons/ ha)		Berry weight (g/berry)	Cluster weight (g/ cluster)
Unthinned control	2012	19.1 $\pm$ 0.7	a	50.9 $\pm$ 2.0	a	1.58 $\pm$ 0.09	178.9 $\pm$ 2.4
50 % cluster thinning	2012	10.6 $\pm$ 0.2	b	28.4 $\pm$ 0.6	b	1.55 $\pm$ 0.10	190.8 $\pm$ 23.1
75 % cluster thinning	2012	7.1 $\pm$ 1.4	c	18.9 $\pm$ 3.7	c	1.64 $\pm$ 0.05	204.1 $\pm$ 8.6
Unthinned control	2013	13.2 $\pm$ 0.7	a	35.2 $\pm$ 1.8	a	1.34 $\pm$ 0.13	150.5 $\pm$ 21.7
50 % cluster thinning	2013	11.6 $\pm$ 1.3	ab	30.8 $\pm$ 3.6	ab	1.40 $\pm$ 0.09	175.3 $\pm$ 13.7
75 % cluster thinning	2013	7.1 $\pm$ 0.5	c	18.9 $\pm$ 1.3	c	1.51 $\pm$ 0.13	183.2 $\pm$ 27.7
Unthinned control	2014	17.3 $\pm$ 2.6	a	46.3 $\pm$ 6.9	a	1.52 $\pm$ 0.17	166.2 $\pm$ 4.2
50 % cluster thinning	2014	7.7 $\pm$ 1.1	b	20.5 $\pm$ 2.8	b	1.60 $\pm$ 0.20	147.4 $\pm$ 22.4
75 % cluster thinning	2014	3.5 $\pm$ 1.1	b	9.2 $\pm$ 2.9	b	1.68 $\pm$ 0.08	165.6 $\pm$ 19.9

2.4.2. Berry attributes profiling

The average weights of berries were determined at each time point by weighing 10 berries from 10 of the 26 different clusters collected from each vine block. For each sample, the juice was analyzed for standard berry chemical attributes: reducing sugar (glucose–fructose kit GL7954; UV Method; Randox, Antrim, UK) and malic acid (L-malic acid kit ML7314/7315; UV Method; Randox, Antrim, UK). The glucose–fructose testing employed hexokinase and glucose-6-phosphate dehydrogenase enzymes to convert glucose to gluconate-6-phosphate and producing NADH. The presence of the phosphoglucose isomerase enzyme (Sigma-Aldrich, St Louis, MO, USA) converted fructose-6-phosphate to glucose-6-phosphate making it available to react as well. The increase in NADH, directly proportional to glucose concentration, was measured at 340 nm. Malic acid testing employed the malate dehydrogenase enzyme, which converted malic acid to oxaloacetate producing NADH. The increase in NADH was measured at 340 nm and considered directly proportional to malate concentration. Berry attributes were plotted by DAV using the smoothed conditional means function (level of confidence = 0.95) of the R package ggplot2 version 3.4.1 (Wickham, 2009).

2.4.3. Grape chemistry at harvest

Grape chemistry was analyzed at harvest. Grapes (20 of the 26 collected clusters) were destemmed and homogenized using a Vitamix blender (Vitamix Corporation, Cleveland, OH).

The total phenol content of grape homogenates was analyzed using the Folin-Ciocalteu Assay (Singleton et al., 1999), and the composition of phenolic compounds was assessed using an Agilent 1200 reversed-phase high-performance liquid chromatography (HPLC) system equipped with a diode array detector and a 4.6 $\times$ 50 mm Zorbax Eclipse Plus C18 column (Agilent Technologies Inc., CA). HPLC was performed using a gradient mobile phase system of acidified water (0.2 % v/v phosphoric acid) and acetonitrile at a flow rate of 1 ml/min, as described by Waterhouse et al. (1999). Monomeric polyphenols were quantified using authentic reference standards at the following absorbances: gallic acid, catechin, and epicatechin (280 nm); quercetin, quercetin, and quercetin glycoside (360 nm); and malvidin (520 nm). Polymeric tannins were quantified against absorbance of a catechin reference standard measured at 280 nm, and pigmented polymers were measured using a reference standard of malvidin-3-diglucoside, measured by absorbance at 520 nm (Previtali et al., 2021).

A WineScan FT-120 Fourier transform infrared (FTIR) spectroscopy system (FOSS North America, MN) was used to determine total soluble solids, pH, titratable acidity (TA) and malic acid. FTIR calibration functions for these parameters were developed using standard primary analytical methodologies, like described by Kupina and Shrikhande (2003).

2.4.4. Grape metabolomic profiling

Sixty berries, from six isolated clusters randomly selected from the 26 collected from the vine blocks, were ground under liquid nitrogen. Seeds were removed before grinding. Frozen powder was divided into 100-mg aliquots for UHPLC-QTOF-MS analysis.

Samples were fractionated using an Agilent 1290 UHPLC-6530-QTOF and an Agilent 1290 UHPLC-6550-QTOF equipped with a Acquity CSH C18 2.1 $\times$ 100 mm 1.7 μ m column and a Acquity VanGuard CSH C18 1.7 μ m pre-column (Waters Corp., Milford, MA, USA) and were analyzed by quadrupole time-of-flight mass spectrometry (QTOF-MS) in positive and negative ion modes. Two solvents were used for separation: 100 % acetonitrile in water (solvent A) and 100 % isopropanol (solvent B). After injecting 1.67 μ l of sample at a flow rate of 0.5 μ l/min, a solvent gradient was established from 85 % to 1 % solvent A in 12 min and from 15 % to 99 % solvent B in 12 min, followed in each case by a 3-min equilibration. The retention time, intensity, mass accuracy and peak width of analytes were monitored and matched to a reference library leading to the putative identification of 72 metabolites, including

flavan-3-ols, flavan-3-ol oligomers, phenolic acids, flavonol and dihydroflavonol glycosides, and anthocyanins. The dataset was normalized using the median scaling approach to reduce the batch effect across vintages (Reisetter et al., 2017). Among all assigned metabolites, we focused on 16 phenolic compounds and group them in seven metabolite classes (Total, 3'5'-OH and 3'-OH anthocyanins; Flavan-3-ols; Flavonols; Hydroxycinnamic and benzoic acid derivatives; Stilbenes) to better depict phenolic profiling by crop load condition.

The effect of crop load on the accumulation of specific classes of phenolic compounds was evidenced plotting their quantification (in arbitrary units, AU) by crop load category (balanced, overcropped, undercropped) over time (DAV) using the smoothed conditional means function of the R package ggplot2 version 2.2.1 (Wickham, 2009). This R function allowed setting a level of confidence (0.95) to evidence the significant differences.

2.5. Gene expression analysis

2.5.1. RNA extraction

The same powdered samples used above for metabolomic analysis were divided into 400-mg aliquots for RNA extraction. Total RNA was isolated using the Spectrum Plant Total RNA kit (Sigma-Aldrich, St Louis, MO, USA) following the manufacturer's instructions with some modifications (Fasoli et al., 2012). RNA quality and quantity were determined using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and a Bioanalyzer Chip RNA 7500 series II (Agilent).

2.5.2. Library preparation and RNA sequencing analysis

We prepared 255 non-directional cDNA libraries from 2.5 µg total RNA using the Illumina TruSeq RNA Sample preparation protocol (Illumina Inc., San Diego, CA, USA). Library quality was determined using the Agilent High Sensitivity DNA kit on the Agilent 2100 Bioanalyzer, and the quantity was determined by quantitative PCR using the KAPA Library Quantification kit (Kapa Biosystems, Roche Diagnostics, Basel, Switzerland). Single-end reads of 100 nucleotides were obtained using an Illumina HiSeq 1000 sequencer and sequencing data were generated using the base-calling software Illumina Casava v1.8. Chastity filtered reads were aligned to the grapevine 12x reference genome PN40024 (Jaillon et al., 2007) using TopHat v2.0.6 with default parameters (Kim et al., 2013). Mapped reads were used to reconstruct the transcripts using Cufflinks v2.0.2 (Roberts et al., 2011) and the reference genome annotation V1 (<https://grapedata.org/genomes/>). The normalized expression of each transcript was calculated as reads per kilobase of transcript per million mapped reads (RPKM) for each sample. Transcriptomic data is presented using the V3 genome functional annotation (VCost.v3; Canaguier et al., 2017).

2.6. Data analysis

2.6.1. Correlation analysis

To integrate berry transcriptome and crop load information, we selected by functional annotation the 383 phenylpropanoid pathway related genes defined expressed (average expression value >1 RPKM across the entire dataset) and compared their profiles with the crop load estimation by Ravaz Index (RI) using a correlation matrix (Spearman metric).

2.6.2. Linear mixed effect model

Linear mixed models were used to explore differences in gene expression patterns by varying crop loads (Ravaz Index) and in order to treat vintage as a random effect (Vink et al., 2017). Using linear mixed effects models to analyze longitudinal gene expression can highlight differences between sample groups over time, i.e. differences by crop load over the course of berry development. For each of the 29,971 genes, a mixed model was fitted with gene expression as dependent variable

and crop load status (by RI) as an independent variable. Fixed-effect covariates included in the final model were time (DAV) and sugar accumulation (RS). Mixed models and p-values were computed using the R function lmer from the R package lme4 (Bates et al., 2015). To correct for multiple testing, a Bonferroni correction was applied ($p < 0.05$).

2.6.3. Gene clustering

The clustering analysis of LME-significant genes was performed using the hclust function of R. Pearson's correlation and the complete linkage were chosen as the distance metric and the clustering method, respectively. The number of significant gene clusters was evaluated by the Within groups sum of squares using the k-means function of the R package cluster (Kaufman and Rousseeuw, 1990).

2.6.4. Analysis of variance

One-way analysis of variance (ANOVA) with Tukey's honest significant difference (HSD) test was applied to evaluate the significance of variation in field data and berry attributes and composition by treatment or crop load level ($p < 0.05$), using the online tool at "Online Web Statistical Calculators" (<https://astatsa.com/>).

2.6.5. GO enrichment analysis

All grapevine transcripts were originally annotated against the V1 version of the 12 × annotation of the grapevine genome and later intersected with the V3 update of the genome functional annotation (VCost.v3; Canaguier et al., 2017; <https://grapedata.org/genomes/>). GO annotations were assigned using the BiNGO version 2.3 plug-in tool in Cytoscape (<http://www.cytoscape.org/>) version 2.6 with PlantGoslim categories. Overrepresented PlantGoslim categories were identified using a hypergeometric test with a significance threshold of 0.05 (Klipper-Aurbach et al., 1995).

3. Results

3.1. Crop load manipulation in Pinot noir

To investigate the effect of crop load on Pinot noir berry development and maturation, moderate and severe cluster thinning treatments were carried out immediately after fruit set to remove respectively 50 % and 75 % of the bunches on each vine. Treatments were compared to unthinned vines over three consecutive vintages.

The yield at harvest varied by vintage: 2012 and 2014 seasons recorded an average tonnage of 50.9 and 46.3 tons/ha respectively, whereas 2013 vintage featured a lower production (35.2 tons/ha; Table 1 and Supplemental Dataset S1). Upon cluster thinning, yield was reduced by 44 % and 63 % in 2012, by 12 % and 46 % in 2013, and by 56 % and 80 % in 2014, respectively for the moderate and severe treatments. The treatments did not seamlessly meet the targeted 50 % and 75 % crop reduction for all years and cluster thinning was less effective in 2013. The average bunch and berry weight did not significantly vary by treatment (Table 1 and Supplemental Dataset S1).

We collected berry samples from fruit set to full maturity every 7–10 days using a randomized block approach to account for intra-vineyard block variability resulting in a collection of 255 samples (Supplemental Dataset S2). The recording of heat accumulation (growing degree days; Fasoli et al., 2018) showed that the 2013 and 2014 seasons were warmer than 2012 from March to August and veraison (defined as the 50 % colored berries per cluster), therefore, occurred 10–20 days earlier (Fig. 1 and Supplemental Dataset S1). Also, the 2013 season was characterized by drier conditions than the other vintages (Fasoli et al., 2018). The cluster thinning treatments determined modifications of the development of the fruit that appeared as both anticipation of the onset (veraison) and acceleration of the maturation phase. Moderate thinning determined a two-day shift of the veraison dates in 2012 and 2014 compared to the controls but did not have any effect in 2013, whereas vines treated with severe cluster thinning exhibited a stronger effect and

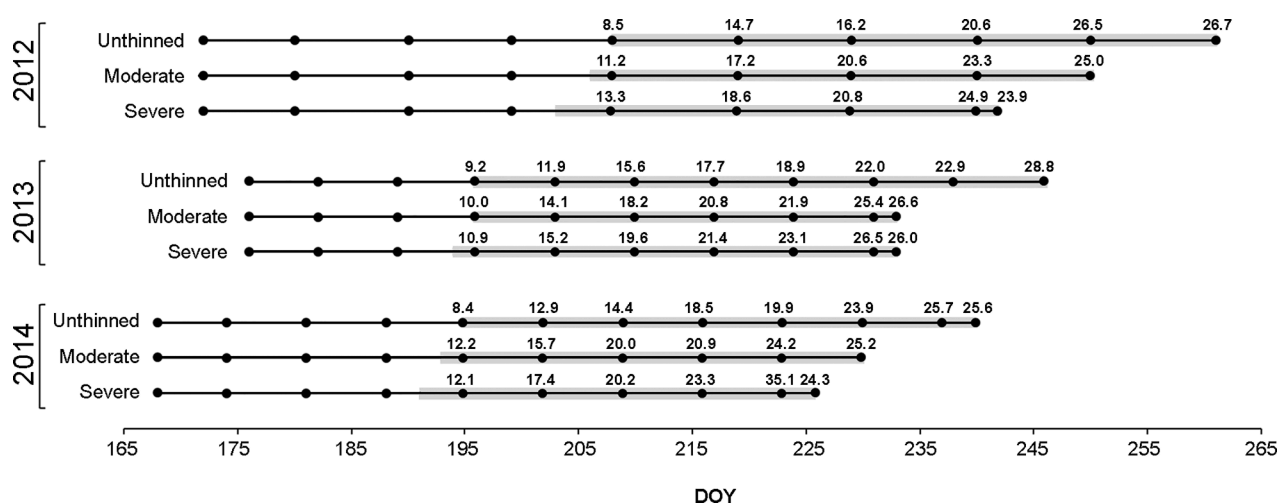


Fig. 1. Pinot noir time series of berry sample collection. Berries were collected from control (0 %), 50 % (50 %) and 75 % (75 %) thinned vines in 2012, 2013 and 2014 using a randomized block approach to account for intra-vineyard block variability. This resulted in a collection of 255 samples. (●) sampling time points; the gray bars indicate the time window from veraison to harvest. Brix values are specified for time points post veraison along the timeline. Veraison: visually defined as the 50 % colored berries per cluster. DOY, day of the year.

veraison dates ranged from four days early in 2014, to five and seven in 2012 and 2013 respectively (Fig. 1 and Supplemental Dataset S1). The impact of cluster thinning on the beginning and the course of maturation determined earlier commercial maturity and harvest dates for the treated vines compared to the unthinned (Fig. 1 and Supplemental Dataset S1). Berry composition at harvest was significantly influenced by cluster thinning in 2012 (Tukey's test, $p < 0.05$), whereas it did not show any significant difference in the following vintages for any of the tested attributes (Table 2). Although ripening progression was monitored to meet identical maturation level by TSS, the fruit displayed variations due to the biological variability present intra vineyard. The most relevant difference was recorded for the severe cluster thinning that, in line with lower soluble solids, was associated with more acidic berries. In this particular case, the harvest decision was likely made a few days earlier than optimal in order to achieve the desired technological maturity level. However, this difference does not likely relate to the vintage climatic conditions nor to the experimental treatment effects being studied.

The effect of cluster thinning on crop load was evaluated and interpreted using the RI, namely comparing the yield at harvest to the dormant pruning weight measured in the winter succeeding harvest (Fig. 2A, Supplemental Table S1 and Dataset S1). The ratio between fruit yield and vine size varied across the experiments at harvest, with a decrease being clearly related to the extent of the thinning. However, only in 2012 the decrease in the yield/pruning weight ratio was nearly proportional to the treatments, with a significant distinction across the RI values. The effect was less evident in 2013, showing a significant difference only between control and extreme thinning ($p < 0.05$), and

2014 when the extent of cluster thinning led to a significant difference in RI between control and treatments ($p < 0.01$) but not between treatments. To evaluate the effect of the different sink-to-source balancing on berry development, we defined three crop load levels (Kliever and Dokoozlian, 2005): undercrop (RI < 5), balance (5 < RI < 8), and overcrop (RI > 8). Unthinned vines generally resulted in the overcrop category, except one replicate in 2013 that, in the subsequent computational steps, was assigned to the balanced state group (Supplemental Dataset S1). Moderate cluster thinning (50 % crop production) determined the decrease of the yield/pruning weight ratio to the balance condition in 2012 and 2013, and to the undercrop state in two out of three replicates in 2014 hence determining the classification of the related samples as undercrop to polish the data analysis and interpretation. Severe thinning (75 % crop reduction) generally determined the achievement of undercrop condition, except for one sample replicate in 2013 that fell into the balance crop load category and then was also re-assigned to the matching condition (i.e., balance instead of undercrop).

To minimize vintage and cluster thinning effects on the onset of ripening, in the following comparison of technological, compositional and molecular ripening parameters, fruit development and maturation series were aligned by DAV. Yet, the sugar accumulation trends showed the effect of the treatments that, at lower crop loads, consists of an increased maturation rate (Fig. 2B). On the other hand, malic acid depletion and berry weight profiles did not show a clear effect by crop load (Supplemental Figure S1).

Table 2

Berry composition at harvest by cluster thinning level. The technological maturity of grapes sampled from each treatment was determined by defining the total soluble solids, pH, titratable acidity (TA) and malic acid. Averaged values ($n = 3$) $\pm$ standard deviation are reported. Different letters to the right of the numbers denote significant differences by treatment within each vintage (Tukey's test, $p < 0.05$).

Treatment	Year	Total soluble solids (°Brix)	pH	Titratable acidity (g/l)	Malic acid (g/l)
Unthinned control	2012	24.90 $\pm$ 0.10	a	3.46 $\pm$ 0.01	a
50 % cluster thinning	2012	24.77 $\pm$ 0.59	ab	3.47 $\pm$ 0.01	a
75 % cluster thinning	2012	23.93 $\pm$ 0.21	b	3.38 $\pm$ 0.02	b
Unthinned control	2013	27.23 $\pm$ 1.34	3.57 $\pm$ 0.09	0.49 $\pm$ 0.07	2.07 $\pm$ 0.52
50 % cluster thinning	2013	24.90 $\pm$ 1.59	3.41 $\pm$ 0.11	0.61 $\pm$ 0.10	2.86 $\pm$ 0.56
75 % cluster thinning	2013	24.63 $\pm$ 1.45	3.42 $\pm$ 0.06	0.65 $\pm$ 0.10	3.08 $\pm$ 0.57
Unthinned control	2014	24.27 $\pm$ 1.10	3.49 $\pm$ 0.04	0.54 $\pm$ 0.02	2.98 $\pm$ 0.57
50 % cluster thinning	2014	23.80 $\pm$ 0.40	3.64 $\pm$ 0.08	0.58 $\pm$ 0.06	3.72 $\pm$ 0.19
75 % cluster thinning	2014	23.87 $\pm$ 0.38	3.59 $\pm$ 0.07	0.61 $\pm$ 0.02	3.04 $\pm$ 0.19

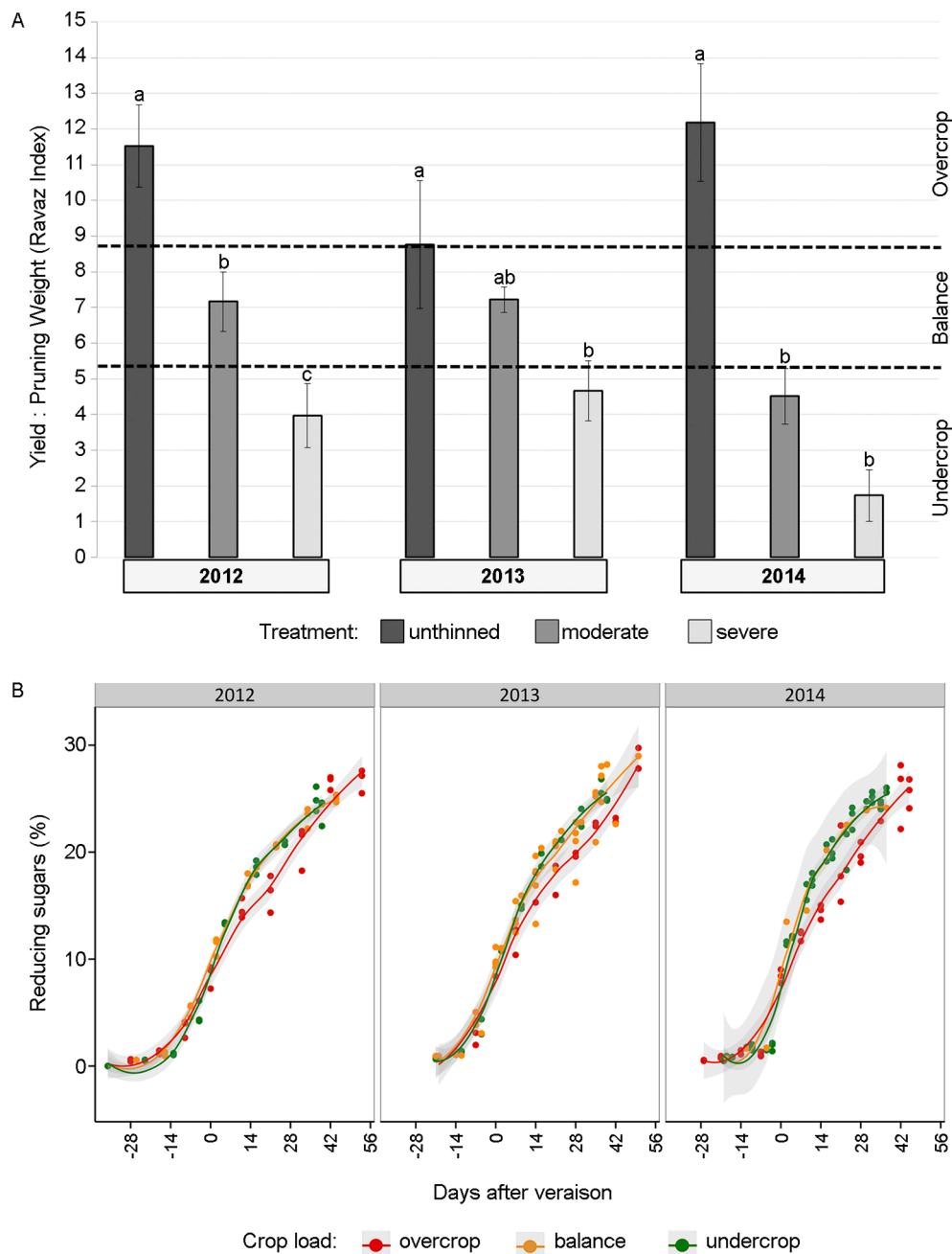


Fig. 2. Harvest and berry development data. A, Yield to pruning weight (Ravaz Index) results across vintage 2012, 2013 and 2014, for each treatment. Dashed lined mark the definition of crop load categories: Ravaz Index (RI) > 8 represents overcrop condition, $5 < \text{RI} < 8$ corresponds to vine balance and $\text{RI} < 5$ defines undercrop vine state. Different letters above plot bars denote significant differences by treatment within each vintage (Tukey's test, $p < 0.05$). B, Grape berry development is shown by reducing sugar accumulation from fruit set to harvest by crop load category. Line graphs were created distinguishing data from each vintage and plotting by days after veraison. Gray shading indicates 0.95 confidence levels relative to the smoothed conditional means plotting method.

3.2. Analysis of crop load effect on phenolics

The accumulation of phenolic compounds was analyzed over time and compared across the three crop load levels (Supplementary Dataset S3). Total anthocyanins accumulated more rapidly and to a greater extent in balanced and undercropped berries compared to overcropped (Fig. 3A). The accumulation trend over time highlighted that the anthocyanin content peaked around 28–42 DAV and tended to decrease in concentration at late ripening stages, in particular at harvest in 2014. Metabolomic analysis revealed that the increase in total anthocyanins was evenly distributed among the five main glycosylated species that characterize the Pinot noir cultivar (Supplemental Figure S2A).

However, the glucosylated form of peonidin showed the most significant increase in the balance condition compared to over and undercropping.

Stilbenes content was also influenced by crop load (Fig. 3B). Measured stilbenoids were resveratrol and its dimers (pallidol or viniferin). Resveratrol accumulation started after veraison, whereas its polymerization began later, showing very little contribution to the overall stilbenes content (Supplemental Figure S2B). Total stilbenes accumulation was enhanced in overcrop conditions, although exhibiting a downward trend towards the later sampling time points in vintage 2014. Stilbenes content at harvest ended up being comparable between overcrop and balance vine status, while undercropped vines maintained lower content. The scatterplot of total anthocyanins by stilbenes values

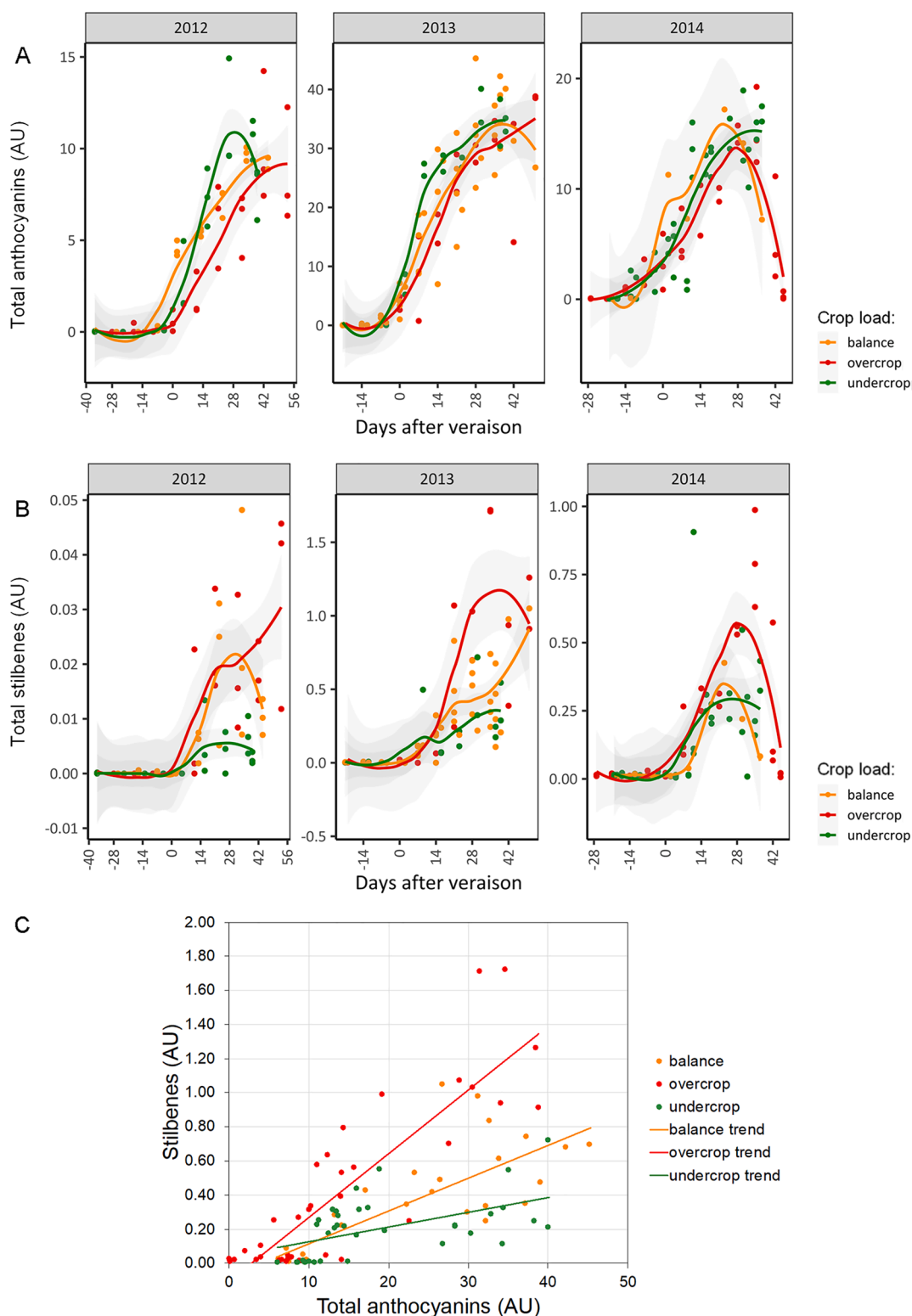


Fig. 3. Phenylpropanoid accumulation trends. A, B Total anthocyanins (A) and total stilbenes (B) accumulation profiles show distinguishable trends by varying crop load. Line graphs were created distinguishing data from each vintage and plotting by days after veraison. Gray shading indicates 0.95 confidence levels relative to the smoothed conditional means plotting method. Normalized metabolite content is defined by Arbitrary Unit (AU). C, Scatterplot of total anthocyanins by stilbenes values in post-veraison berries. Data from three vintages were used. Linear trendlines evidence changes in the ratio between the two class of metabolites by crop load category.

in post-veraison berries (from time point 6 to harvest) shows that the stilbenes:anthocyanins ratio changes at varying crop load conditions (Fig. 3C). The linear trendlines evidence that overcrop condition

features a greater stilbenes:anthocyanins ratio, whereas reduced RI favors a smaller ratio with the undercrop condition showing little increase of stilbenes content over time.

Total anthocyanins and additional phenolic compounds, namely total polymeric tannins and the flavonol quercetin-3-O-glucoside that can contribute to mouthfeel characteristics of the wine (Hufnagel and Hofmann, 2008), were quantified at harvest and distinguished by crop load level (Supplementary Table S2). Crop load affected the harvest

concentration of anthocyanins in 2012 and 2014, and of polymeric tannins only in 2012 (Tukey's test, $p < 0.05$). No crop load effect could be distinguished for quercetin-3-O-glucoside concentrations at harvest (Tukey's test, $p < 0.05$).

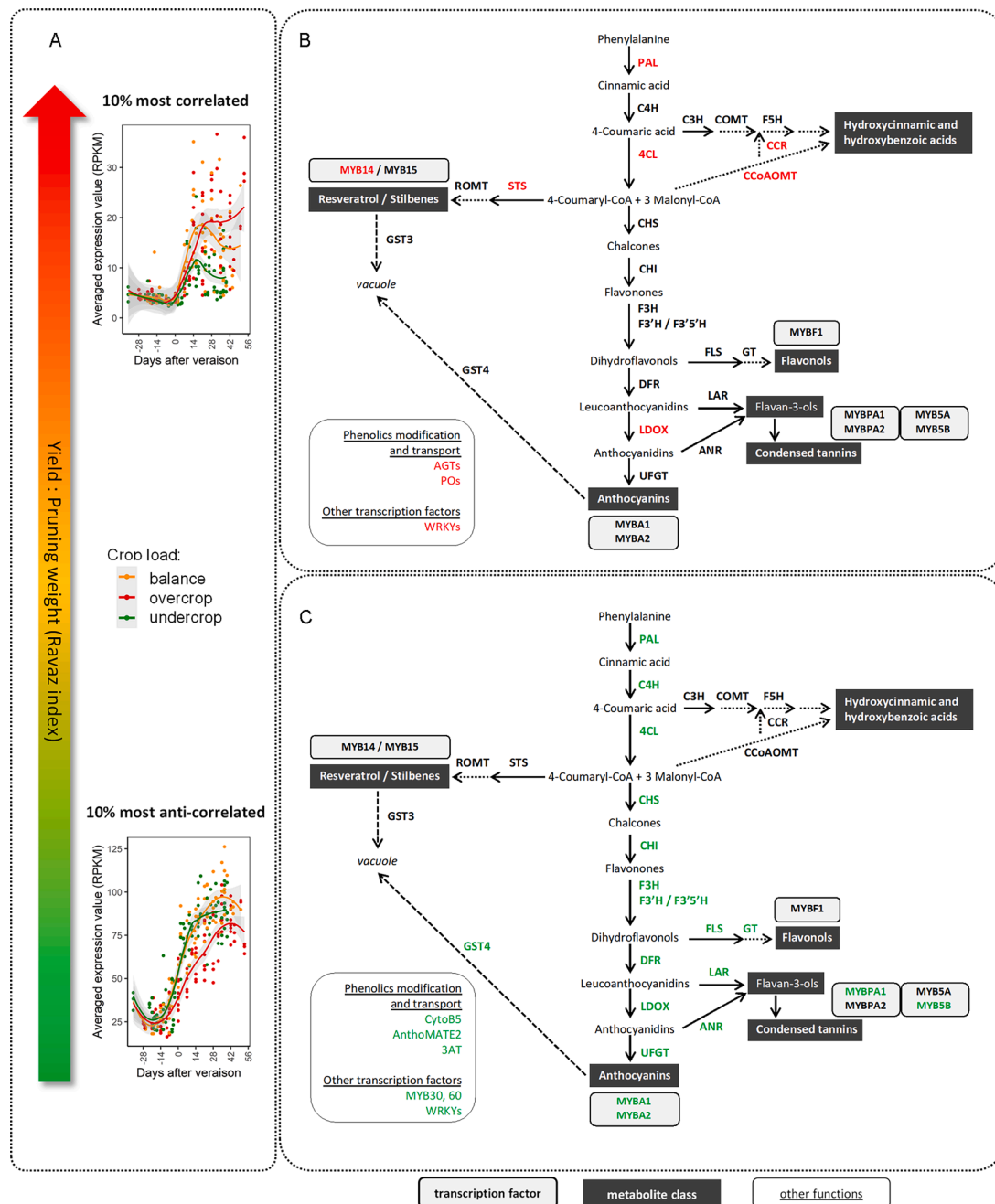


Fig. 4. Correlation analysis between Ravaz index (RI) and gene expression changes in the phenylpropanoid pathway. A, The arrow indicates RI increase (green is low, red is high). The line plots show the averaged expression profile of the 10 % most correlated (top left) and anti-correlated (bottom left) genes to the RI. Line graphs were created using data from three vintages plotted by days after veraison. Gray shading indicates 0.95 confidence levels relative to the smoothed conditional means plotting method. B, C Pathway schematics (B and C respectively for the 10 % most correlated and anti-correlated genes to the RI) show the correlation of specific genes to RI (green font: anti-correlated; red font: correlated; black font: no correlation found). Abbreviations: PAL, phenylalanine ammonia-lyase; C4H, cinnamate-4-hydroxylase; 4CL, 4-coumarate CoA ligase; C3H, coumarate 3-hydroxylase; F5H, ferulate 5-hydroxylase; CCR, cinnamoyl-CoA reductase; COMT, caffeic acid O-methyltransferase; CCoAOMT, caffeoyl/CoA-3-O-methyltransferase; STS, stilbene synthase; ROMT, trans-resveratrol di-O-methyltransferase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3'H, flavonoid-3'-hydroxylase; F3'5'H, flavonoid-3'5'-hydroxylase; FLS, flavonol synthase; GT, glycosyltransferase; F3GT, flavonoid-3-O-glycosyltransferase; DFR, dihydroflavonol reductase; LDOX, leucoanthocyanidin dioxygenase; LAR, leucoanthocyanidin reductase; ANR, anthocyanidin reductase; UFGT, UDP glucose-flavonoid 3-o-glucosyltransferase; GST, glutathione S-transferase; AGT, anthocyanidin 3-O-glucosyltransferase; PO, polyphenol oxidase; WRKY, WRKY transcription factor; CytoB5, cytochrome b5 DIF-P; AnthoMATE2, anthocyanin MATE efflux family protein; 3AT, 3 anthocyanin acyl-transferase.

3.3. Transcriptomic focus on the phenylpropanoid pathway by varying crop load level

RNA-Seq was used to monitor the expression of all grapevine genes (Supplementary Dataset S4) and, to better understand the influence of crop load on the metabolic profiles described above, we focused on the expression of the 858 genes belonging to the shikimic acid and the general phenylpropanoid pathways. The VitisNet annotations were used to assign grapevine genes to functional networks and pathways (Grimplet et al., 2009, 2014). This list also included gene families associated with shikimic and phenylpropanoid pathway transcriptional regulation (MYB and WRKY transcription factors; Holl et al., 2013; Wang et al., 2014; Amato et al., 2016) and phenolic compounds transport (Glutathione-S-Transferases and AnthoMATE transporters; Conn et al., 2008; Gomez et al., 2009; Giordano et al., 2016). We detected 388 genes showing an average expression value >1 RPKM across the subset of genes involved in the two pathways (Supplemental Dataset S5).

To confirm the berry composition data showing that variation in crop load levels may preferentially stimulate different branches of the general phenylpropanoid pathway, we performed a correlation analysis between the RI indices calculated for each sample series and the transcriptomic changes of the 388 genes during Pinot noir berry development and ripening across the three vintages. The 10 % most negatively and 10 % most positively correlated genes were selected from the correlation matrix (Supplemental Dataset S5) and their averaged trends are depicted in Fig. 4A. The pathway schematics (Figs. 4B and 4C) highlight the detail of the correlation to RI for each biosynthetic and regulative step. Among the genes expressing at greater levels as crop load increases towards an overcropping status (Fig. 4A, top plot), we found those belonging to the stilbenes branch, indicating that this group of phenylpropanoids is positively correlated to crop load like suggested by the stilbenes accumulation trend (Fig. 3B). On the other hand, genes related to the chalcones and flavonoids branch seems to be positively regulated at lower crop loads, which indeed mirrors the greater production of anthocyanins when vines are in undercrop and balance conditions (Fig. 3A). Key genes of the shikimic pathway like 3-DEOXY-D-ARABINO-HEPTULOSONATE 7-PHOSPHATE SYNTHASE 3 (DAHPS3) and SHIKIMATE KINASE 2 (SK2) (not represented in the schematic pathway of Figure 4; see Supplemental Dataset S5) are influenced positively by lower crop loads. DAHPS4, conversely, shows a greater peak of expression at overcrop and balance condition (Supplemental Figure S3A). Vine crop load influence on the berry secondary metabolism at the transcriptional level becomes more evident downstream and encompasses the phenylpropanoid biosynthesis from the precursor phenylalanine produced by the shikimic pathway. The families of the early genes of the pathway upstream the crossroad between stilbenes and chalcones (PAL, 4CL) seem to be split between over and undercrop-related (Fig. 3 and Supplemental Figure S3B). Flavonoids and anthocyanins related genes, like LEUCOANTHOCYANIDIN DIOXYGENASE 1 (LDOX1) and UFGT1, were listed among the negatively correlated to crop load, namely their profiles showed a higher expression level at undercrop and balance compared to overcrop vine status. LDOX1 showed the main difference by crop load when peaking around veraison, whereas UFGT1 exhibited higher values at lower crop loads over the entire ripening phase (Supplemental Figure S4A). Conversely, crop load positively correlated genes had greater expression values at overcrop and balance status, such as MYB14 transcription factor and 27 stilbene synthase genes (see Supplemental Figure S4B where STS7 serves as an example of the stilbene synthase gene family behavior).

3.4. Study of the effect of crop load on berry transcriptome at the onset of ripening

An LME model was used to unravel the gene expression patterns significantly modulated by crop load within the veraison time frame. For each of the 29,971 genes, a mixed model was fitted with gene expression

as dependent variable and crop load status (by RI index) as independent variable. Fixed-effect covariates included in the final model were time (DAV) and reducing sugar (RS) accumulation. To account for climatic variability, vintage was figured as random effect. Mixed models and p-values were computed using the R function lmer from the package lme4. To correct for multiple testing, a Bonferroni correction was applied ($p < 0.05$). The model was applied to a reduced set of samples to focus on the molecular events happening around the onset of ripening (between 9 days before and 26 after veraison; Fig. 5, top), namely comprising the time points 3–6 of all vintages. We found 1030 genes that showed significant modulation by crop load and time in the selected interval (Supplemental Dataset S6), therefore genes influenced by crop load when participating to the berry developmental program. Clustering analysis of the 1030 genes over the whole fruit development highlighted four main distinctive trends (Fig. 5). The expression of the genes belonging to each cluster was averaged to allow a more detailed visualization of the profiles over the entire berry development and visualize the variation associated to crop load. The first derivative of each averaged gene expression trend ($\Delta\text{RPKM}/\Delta t$) was also computed for the time window -9 to 26 DAV aiming at emphasizing the effect of crop load on the rate of the molecular changes at the onset of ripening, i.e., gene expression escalation at specific conditions and time.

Cluster 1 includes the majority of the differentially expressed genes at the time window analyzed (803 genes) that have a sharp downward trend that reaches its minimum by two weeks after veraison for all vine statuses. The crop load effect on the expression of these genes is weakly apparent at veraison when overcrop condition causes a subtle delay in reaching the minimum transcription. However, the effect of crop load is evidenced distinctly by the first derivative: genes at balance and undercrop conditions (almost overlapping along the whole-time frame) decrease their expression much faster as veraison is approached. GO enrichment analysis showed that genes belonging to the photosynthesis, generation of precursor metabolites and energy, response to abiotic and endogenous stimuli categories were significantly overrepresented ($p < 0.05$; Supplemental Dataset S6). In fact, genes encoding photosynthetic and cell respiration components are highly represented in this group, in addition to transcripts related to auxin (i.e., the INDOLE-3-ACETIC ACID-AMINO SYNTHETASE GH3.2, Supplementary Figure S5A; Dal Santo et al., 2020), ethylene and ABA response and signaling. Transcription factors like the putative MADS-box AGAMOUS 2 and FRUIT-FULL 2 (AG2 and FUL2, Supplementary Figure S5A; Grimplet et al., 2016), the AQUAPORIN TMP-C and other transporters also appeared in cluster 1.

A similar acceleration of the developmental program at lower crop loads characterizes the behavior of the 38 genes grouped in cluster 2, peaking around veraison and on average reaching greater expression in undercrop and balance conditions. In fact, despite the curves appearing fairly aligned by DAV, the variation of RPKM by time spotlights that the upregulation and subsequent downregulation of these genes are much faster at lower RI values. Cluster 2 includes genes encoding for transporters like GLUCOSE-6-PHOSPHATE/PHOSPHATE TRASLOCATOR and TONOPLAST MONOSACCHARIDE/HEXOSE TRANSPORTER (Afoufa-Bastien et al., 2010), for an ALCOHOL DEHYDROGENASE, and for several proteins related to cell wall metabolism, such as EXPANSIN A1 and A18 (EXPA1 and EXPA18, Supplementary Figure S5B; Dal Santo et al., 2013).

The other two clusters include genes depicting a simple delay of their expression pattern in overcrop condition, without a meaningful acceleration or deceleration of their modulation. The 135 genes included in cluster 3 gradually decrease their expression until veraison, then show a steep upward trend maintained to harvest. The profiles by crop load status appear clearly shifted, especially for the overcrop condition and from veraison onward. Zooming into the analyzed time window with the first derivative highlights that the balance condition determines a slightly faster decrease of expression before veraison. No biological process was enriched for this group of genes, however it is worth

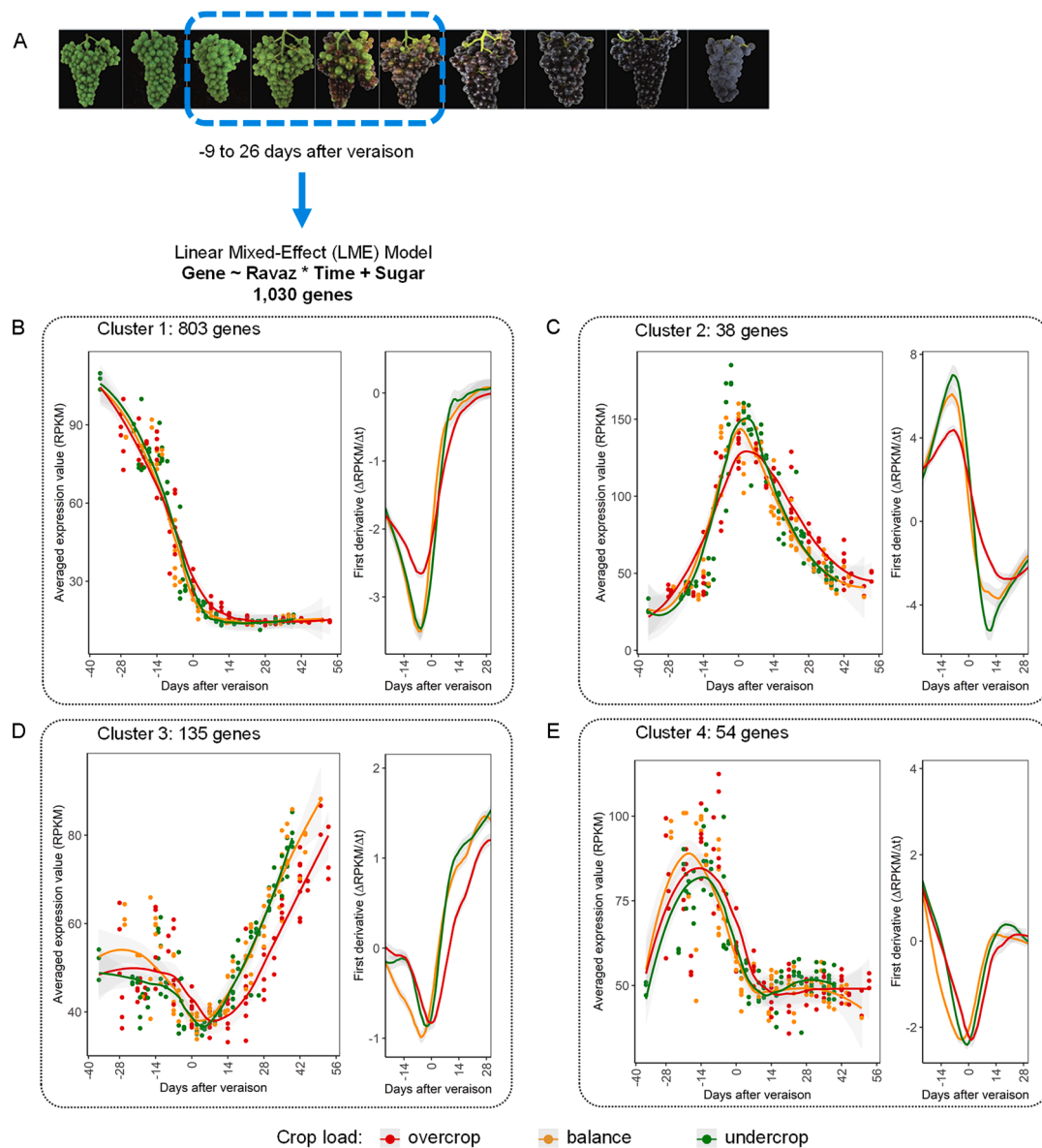


Fig. 5. The effect of crop load on putative veraison-related gene expression patterns. A, schematic highlighting the time window investigated using the LME approach (−9 to 26 days after veraison). The four selected photos depict clusters at pre-veraison/green stage and at the beginning, 70 % and 95 % of berry coloring (from left to right). Veraison is defined as the 50 % colored berries per cluster. B, C, D, E, Clusters of gene expression patterns identified by hierarchical cluster analysis applied on 1030 significant modulated genes. Data from three vintages were used. Gene expression trend (left panels) and its first derivative (right panel) are plotted by days after veraison.

mentioning that 29 genes related to transcription factor activity are listed, like the *LATERAL ORGAN BOUNDARIES (LOB) DOMAIN class I α3* gene (*LBD1a3*, Supplementary Figure S5C; [Grimplet et al., 2017](#)).

Cluster 4, which groups 54 genes, shows a peak of expression before veraison (−14 DAV) followed by a steep downward trend to reaching a minimum value (at ~14 DAV) that is maintained until harvest. At the onset of ripening the shifted expression curves show variation by crop load condition. The derivative plot confirms such shifted behavior and shows that no faster or slower downregulation characterize the behavior of genes for the different crop load levels. This group comprised four genes related to the response to auxin stimulus, seven putative transcription factors and the (9,10) (9',10') *CAROTENE CLEAVAGE DIOXYGENASE 4a* (*CCD4a*, Supplementary Figure S5D; [Lashbrooke et al., 2013](#)).

Overall, this analysis revealed that, despite having re-aligned samples by DAV, still the transcriptional program is influenced by the crop load level, with clear anticipation/delay and acceleration/deceleration

effects.

4. Discussion

4.1. Crop load manipulation by cluster thinning impacts maturation dynamics and composition of Pinot noir grapes

Cluster thinning is a method of pursuing vine balance that can influence berry ripening rate and composition ([Dokoozlian and Hirschfeld, 1995](#); [Pallioti and Cartechini, 2000](#); [Guidoni et al., 2002](#); [Kliwer and Dokoozlian, 2005, 2008](#); [Dokoozlian and Wolpert, 2009](#)).

Our results showed that thinning impacted yield and fruit ripening dynamics in Pinot noir vines in proportion to the crop reduction but at varying extents depending on the vintage. Temperature and rainfall differences recorded across the three year experiment influenced the phenology of the vines as well as the effect of the treatments on berry development itself. Cluster thinning and, in general, source:sink

manipulation effects have been observed as affected by vintage variability in several works (Guidoni et al., 2002; Keller et al., 2005; Nuzzo and Matthews, 2006; Guidoni et al., 2008; Keller et al., 2008; Skrab et al., 2021). Parker et al. (2015) confirmed vintage variability in Pinot noir vines subjected to pre-veraison cluster thinning treatments and observed that removal of sinks via crop thinning became relevant only when source size was substantially reduced by pre-veraison defoliation and likely resulted more limiting. Indeed, the fact that the fruit is only one of several sinks in the vine while the canopy is the main source of photosynthates represents a confounding factor when interpreting and comparing experiments of crop load manipulation. The Pinot noir vines tested in this work could be considered at suboptimal status ($RI > 8$), so the reduction of clusters allowed to bring the plants to either a more balanced range of source:sink ratio ($5 < RI < 8$) or an undercrop level ($RI < 5$). Treatments were less effective in 2013 when, likely because of more extreme climatic conditions (i.e., drought and higher temperatures), production at harvest was lower and, hence, generating a broad range of crop load conditions was more challenging.

Although harvest decisions were based on achieving a target sugar content (24.5 Brix), the changes in fruit development and ripening kinetics determined variations in the composition of the berries at harvest by crop load. Altered rates of sugar accumulation due to manipulation of the source:sink ratio by thinning (clusters or berries) were apparent first (Guidoni et al., 2008; Karoglan et al., 2014; King et al., 2015; Vander-Weide et al., 2024). The detected differences in TSS and acidity of the grapes at harvest were likely due to slightly misaligned sampling developmental stages between treatments. Overall, across the considered vintages, berry composition in terms of the technological maturity (Table 2) was not consistently affected by the crop load manipulation treatments. Cluster thinning had a slight but clear effect on the timing of maturation onset, aspect that has been rarely reported and that here consisted in shifts of the veraison date ranging from two to seven days, depending on the severity of the treatment. The normalization of grape development and maturation time-series by DAV created the opportunity to not only minimize the vintage variable but also dissect the acceleration effect of cluster thinning on berry maturation – that emerges as a temporal shift – from the variations in the activation extent of ripening-related pathways. Lower crop loads exhibited an increased maturation rate, and this trend was distinguishable in the overall ripening-related program both by quality attributes and at molecular level. While this acceleration effect was rather evident when halving the yield of overcropped vines, applying the severe treatment did not further contribute to hasten the maturation program. We can interpret that meeting a balance crop load put the vines at their maximum ripening rate, that cannot be exceeded even at lower crop loads.

4.2. Crop load modulates specific branches of the phenylpropanoid pathway

Manipulations of the source:sink ratio often leads to alteration of the primary metabolism (e.g., related to sugars) as well as of the content and accumulation trends of secondary metabolites, such as anthocyanins, thiols, and phenolic compounds (Stoll et al., 2010; Bobeica et al., 2015; Previtali et al., 2021). Previous research has indicated for red cultivars, such as Refosco and Sangiovese, that crop thinning increases anthocyanins and phenolic substances (Guidoni et al., 2002; Petrie and Clingeleffer, 2006; Sivilotti et al., 2020; Previtali et al., 2021, 2022). The conditions created by thinning of Pinot noir clusters (either balance or undercrop vine status) confirmed an enhancing effect for the total anthocyanins content. Cluster thinning is indeed known to have an overall boosting effect on the entire phenylpropanoid pathway and then to increase the resveratrol content in wine (Prajitna et al., 2007; Reynolds et al., 2007), likewise confirmed by Pastore and coauthors (2011) showing the upregulation of 19 STSs in their microarray data. On the other hand, our findings showed a modulation of the phenylpropanoid pathway depending on the vine crop load status: overcrop condition

featured high stilbenes:anthocyanins ratios, while reduced RI promoted little accumulation of stilbenes content (i.e., low stilbenes:anthocyanins ratio for the undercrop condition). Our comprehensive transcriptomic dataset supports the berry composition results and, based on the correlation analysis, highlighted that the effects of crop load on the phenylpropanoid pathway can be observed at the gene expression level. The pathway appeared to heighten specific branches depending on the vine crop load condition, mostly at the fork between chalcones and stilbenes. Upstream metabolic steps (e.g., PALs, 4CLs) – that are indeed shared by both branches – features the recruitment of different gene family members as regulated in turn with either stilbenes-related or flavonoids-related genes to support the availability of precursors in response to the demand of the downstream steps of the pathway.

Pastore et al. (2011) hypothesized that the synthesis of phenolic compounds such as stilbenes could be part of a systemic response to wounding resulting from the removal of berry clusters, since these compounds are normally produced by the plant in response to stress conditions such as wounding or interactions with pathogens. This however does not explain the difference in the expression of STSs seen in our experiment that, instead, showed the most upregulation in grapes of unthinned and moderately thinned vines. It is possible that applying cluster thinning at veraison retained a stronger wound response over the ripening phase – which is when stilbenes-related genes are being activated – compared to employing treatments at earlier stages like in this work. This might indicate that the enhancement of the stilbene branch is dependent upon the crop load condition established by cluster thinning rather than a direct response to the treatment (wound response). Differences can also be attributed to the varietal features and specific response, as well as in interaction with the environment. Harvest decision was determined by achievement of the target sugar level for all conditions: control unthinned vines were harvested within one to two week later than treated plants consequently their grapes went through the last stages of maturation at dissimilar climatic conditions. Upregulation of the stilbene synthase branch associated to cold conditions was observed in grapevine both during fruit development and grape cluster withering process (Zenoni et al., 2016; Pastore et al., 2017; Shmulevitz et al., 2023). Regardless, the optimal vine status (vine balance) allowed an even regulation of the branches of phenylpropanoid pathway. A balanced crop load seems to be a condition at which the plant does not struggle to channel precursors to the secondary metabolism like can happen at overcrop status when source cannot support all sinks. A balanced vine is more likely capable of developing color and also sustaining a ripening rate that, in Pinot noir, extends into the first week of September, allowing the development of a certain amount of stilbenes, which instead are particularly negatively impacted in undercrop conditions. It is also possible that the stilbene branch is regulated by time and does not depend on either precursors availability nor crop load and only responds to differences in the endurance of the grape ripening process.

4.3. Crop load level influences the onset of ripening in Pinot noir

Crop load ranges determined differences in fruit development and ripening program as a result of the combination of the shift in time of the onset of ripening (i.e., veraison happened earlier at lower crop loads) and of the change in the actual maturation rate. The first factor was minimized – but not zeroed like evidenced in the metabolite and gene plots (Fig. 3) – with normalizing the time series by DAV, whereas the variations in the activation extent of ripening-related pathways were revealed using the LME approach (Fig. 5). To elucidate the earliest effects of crop load manipulation on the grape ripening program, the model was set to scan the molecular events happening between 9 days before and 26 after veraison in all vintages. The LME approach allowed the grouping of genes differentially affected by the crop load level into four clusters of expression profiles. The averaged trends by crop load of the genes belonging to cluster 3 and 4 evidenced a response associated to

the time shift effect of crop load manipulation on the grape development. Hence, it becomes evident that normalizing our dataset with respect to a visual assessment of veraison was not entirely precise and some development-related phenomena results still misaligned. Regardless, the genes assigned to cluster 3 appeared to have the typical profile of the players in the second transcriptional wave during grape berry development that features a higher expression post-veraison, namely during the maturation phase (Fasoli et al., 2018). The group includes the plant and berry switch gene *LBD1a3* (Palumbo et al., 2014; Grimplet et al., 2017) that is expressed at low levels in vegetative/green tissues and show a significant increase in mature/woody organs, suggesting a potential regulatory role during developmental transitions. In particular, *LBD1a3* was identified to be a positive molecular marker of ripening, supported by the presence of cis-acting elements in its promoter that suggests modulation by hormones (Grimplet et al., 2017). Furthermore, both Arabidopsis and banana LBD genes were shown to directly regulate expression of EXPANSIN genes, encoding cell wall-loosening factors (Lee and Kim, 2013; Lee et al., 2013; Ba et al., 2014) that are also modulated during grape ripening.

Interestingly, the other two clusters included genes with a similar timing of downregulation (cluster 1) or transient upregulation (cluster 2) but characterized by a higher rate of modulation associated to the lower crop load levels, as revealed by the analysis of the first derivative of their mean expression profile. This indicates that the crop load level affects the rate at which berries go through the molecular and metabolic changes occurring at the onset of ripening. Cluster 1 describes the shutdown of photosynthetic and cell respiration components and hormonal signaling (auxin-related in particular) typical of the transition of the berry from a vegetative to a ripening phase that, in the case of reduced crop loads, seems accelerated. Our analysis captured the events associated with the decreasing auxin activity, like the expression of *INDOLE-3-ACETATE BETA-GLUCOSYLTRANSFERASES* and *INDOLE-3-ACETIC ACID-AMIDO SYNTHETASES* (*GH3.2*) that resulted in repressed more rapidly in the low crop load condition likewise other genes involved with auxin signaling (Davies and Bottcher, 2009; Dal Santo et al., 2020). Transcription factors of the *MADS-box* family (*AG2* and *FUL2*; Grimplet et al., 2016) represent reminiscences of the functions of the floral homeotic genes controlling flower development. The expression of *FUL2* maps with the carpel-forming region of the flower meristem and continues to be expressed through the early stages of fruit development. *AG2* shows a marked expression peak in the ovary 14 days prior to anthesis, and its transcription tends to decrease onward (Palumbo et al., 2019). Lower crop load appears to be an acceleration factor of the complete transition from flower to fruit identity or rather from the first events of berry formation to the maturation phase.

The role of softening in the initial events of the onset of ripening is remarked by the composition of the cluster 2 (in particular the cell wall-associated *EXPA1* and *EXPA18*; Dal Santo et al., 2013), whose genes – on an average – peaked and then decreased their expression at faster rates when crop load is low and plant status is undercropped or balanced. Moreover, the manipulation of the crop load condition clearly represents a stimulus for sugar translocation as reflected by the modulation of the relative genes (Afoufa-Bastien et al., 2010), which is in line with the idea that when the source:sink ratio is modified the assimilated carbon gets mobilized at different rates depending on the vine status.

In general, some of the genes related to the shutdown of the berry pre-veraison metabolism (cluster 1) and to the activation of the ripening processes (cluster 2) underwent an acceleration of their trends at low crop load levels suggesting a mechanism that conveys and senses the source:sink ratio information in the berry and consequently regulates these crucial molecular events.

5. Conclusions

This study once more confirms that grape metabolism and the berry transcriptome are remarkably flexible, with treatments such as cluster

thinning inducing extensive, genome-wide changes in expression during development. Beyond the major impacts represented by a shift of the onset and completion of ripening, we were able to highlight more subtle effects of the crop load, related to the rate at which the molecular and metabolic changes occur, and to a specific redirection of the phenylpropanoid metabolism affecting the anthocyanin/stilbene ratio. The results support the potential of modifying source:sink ratios as means of optimizing grape yield and quality.

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CRediT authorship contribution statement

Elizabeth Green: Writing – review & editing, Software, Methodology, Investigation. **Ron Shmulevitz:** Writing – review & editing, Investigation. **Alessandra Amato:** Writing – review & editing, Software. **Giovanni Battista Tornielli:** Writing – review & editing, Methodology, Investigation. **Nick Dokoozlian:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization. **Marianna Fasoli:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Investigation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The grapevine berry RNA-Seq data are MIAME compliant and have been deposited in a MIAME-compliant database (Gene Expression Omnibus) at the National Center for Biotechnology Information: control samples can be found under the accession number [GSE98923](#) (Fasoli et al., 2018), whereas cluster thinning related samples under [GSE101532](#). The metabolomics datasets supporting this article are included within the article and its additional files (Supplemental Dataset S3).

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.scienta.2024.113644](https://doi.org/10.1016/j.scienta.2024.113644).

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