EXPLORING THE MECHANISMS OF GRAPEVINE SINGLE BERRY DEVELOPMENT AND RIPENING

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Abstract:

Context and purpose of the study

The strategy of single berry phenotyping is a recently rediscovered research tool that has gained great attention. The latest studies have indicated that previous physiological models based on pooling asynchronous populations of berries provided biased or blurred information on berry development key players. The possibility of monitoring and sampling single synchronized berries to study their development sequentially has opened new lines of research aimed at unraveling the genes that regulate grapevine fruit development. This study aimed to decipher the gene pathways responsible for the activation/deactivation of physiological processes involved in the green phase of growth, the onset of ripening, and the second growth phase.

Material and methods

The growth of single berries of *V. vinifera* cv. 'Syrah' was pictured from flowering until over-ripening through biweekly images in the experimental vineyard of Institut Agro Montpellier, France. Thirty-three single berries (11 developmental phases x 3 biological replicates) were selected for RNA Sequencing based on the relative growth curve and primary metabolite concentrations (sugars and acids) as in Savoi et al. (2021) covering key phases or changes in kinetics during the whole berry development. Specific attention was dedicated to berry softening. Here it is acknowledged as a drop in the fruit tissue firmness and a simultaneous decrease in the glucose/fructose ratio. Gene expressions were normalized and tested for time-series significance leading to twenty-five clusters representing 10,355 genes significantly modulated by development.

Results

Isogenes linked to the phloem sugar unloading pathway, i.e., sugar accumulation, water fluxes, and cell wall remodeling, were confirmed, together with key secondary metabolism regulators. Gene expression profiling clearly showed that these genes were suddenly activated at a specific developmental stage. Great attention was dedicated to the onset of ripening, where precisely with berry softening and, therefore, sugar entry, several genes were switched on and, as intensively, switched off at the maximum berry expansion when phloem stops.
Single berry monitoring allowed distinguishing developmental, molecular events, and critical genes previously considered simultaneous in past transcriptomic studies.

Keywords: Grapevine, *Vitis vinifera*, RNA-Sequencing, Veraison, sugar/acid.

1. Introduction

Grapevine is a prized horticulture crop grown in areas with mild climates, where world-renowned winemaking regions cultivate grapes in areas located between latitudes 30° – 50° and 30° – 40° in the Northern and Southern Hemispheres, respectively. In 2020, the total vineyard surface was 7.3 million hectares for an estimated grape production of 75 million tons per year, with Mediterranean Sea countries being some of the most important worldwide leading viticultural zone (OIV 2021).

Climate change impacts viticulture, with changes in daily range temperature, rainfall patterns, and extreme weather events affecting grapevine growth, grape composition, and wine production (van Leeuwen et al., 2019). Obtaining fully ripe grapes with a low sugar content while maintaining a medium-to-high concentration of malic acid is a modern viticultural challenge imposed by warming, which can only be disentangled with new tools and solutions. So far, the issue has been only partially addressed with ad-hoc vineyard management practices aimed at delaying berry ripening (reviewed in Gutiérrez-Gamboa et al., 2021), such as late- or double-pruning (Palliotti et al., 2017) or leaf removal, although some inconsistent results are reported (De Bei et al., 2019). Therefore, acquiring new knowledge on key players governing the sugar/acid ratio is of utmost importance for identifying the genes responsible for such a particular physiological process. In addition, varietal diversity for berry quality traits exists in the grapevine (Bigard et al., 2022), and once the players are functionally characterized, they can be involved in breeding programs towards an agronomical improvement of elite *V. vinifera* cultivars.

The availability of high-throughput analytical methods and a high-quality draft of the grapevine genome sequence (latest Velt et al., 2022) has led to the characterization of berry development and ripening at the transcriptomic and metabolomic levels (review in Savoi et al., 2022). The development and ripening of grapevine berries are complex processes that involve multiple phases and molecular mechanisms. Berry development follows a double sigmoidal curve with two growth phases separated by a lag phase (Coombe, 1992). During the first growth phase, also known as the green phase, the pericarp enlarges through cell division and expansion, and the berries accumulate organic acids and proanthocyanidins. The lag phase marks the transition to ripening, characterized by the maturation of the seeds. The second growth phase (ripening) involves further changes that make the fruit edible and attractive, such as changes in dimension due to water influx and cell expansion, in texture as berries soften, in palatability because sugars are accumulated while malic acid is respirated, and in colors and aroma thanks to the synthesis of secondary metabolites such as anthocyanins and volatile compounds.

This study aims to phenotypes and transcriptionally characterize fruit development and ripening using advanced berry sampling methodologies that can ensure a quasi-complete synchronization of different berries in a well-defined developmental stage.

2. Material and methods

Experimental design — *Vitis vinifera* cultivar Syrah plants were grown in the experimental facility of the Institut Agro in Montpellier, France. The experiment was conducted during the 2018 season (sampling of berries at phenological stages corresponding to lag and ripening phases) and 2019 (sampling of berries at phenological stages corresponding to green and lag phases). A common phenological point was secured during the lag phase so that the entire developmental cycle of the berry could be reconstructed. The growth of single berries was monitored through recurrent pictures of selected clusters (up to eight) on different plants, and the volumetric growth of single berries was calculated using picture analysis with the ImageJ software.

Primary metabolites analysis — Sugars (glucose and fructose), organic acids (malate and tartrate), and cations (K^+) were analyzed on 100 mg of berry (without seeds) frozen samples of single berries by HPLC as detailed in Savoi et al. (2021), and the net fluxes of water (berry growth), organic acids, and sugar accumulation were calculated for each berry before selecting the one to use as RNAseq samples.

RNA sequencing — RNA extraction as in Rienth et al. (2014) and sequencing were performed on the pericarp of single berry triplicates for each identified developmental stage (11), yielding 33 samples. Samples were sequenced on an Illumina HiSeq3000 in paired-end mode 2x150 bp reads at the Genotoul platform of INRAe-Toulouse.

Transcriptomics analysis — Data analysis involved trimming raw reads for quality and length, aligning reads against the reference grapevine genome PN40024 12X2, counting aligned reads using HTSeq-count, and filtering genes by applying an RPKM>1 cut-off in at least one experimental condition as detailed elsewhere (Savoi et al., 2021). Genes expressed as normalized RPKM were tested for multi-time-series significance using the R package MaSigPro (Nueda et al., 2014) to obtain genes with significant temporal expression changes over berry development and ripening.

3. Results and discussion

3.1 Gene expression analysis over development

A time series RNA-Sequencing approach was adopted to study gene expression over fruit development. The first green growth phase is represented by berries collected 14, 23, and 35 days after flowering (DAF) and characterized by the level of organic acid. Berries sampled at 42 and 47 DAF denoted the lag phase when berry growth slows down, and for this experimental layout, the seasons' juncture. Berries collected at 51 and 54 DAF represented the onset of ripening, specifically at berry softening and a few days later, where no sign of berry re-growth and anthocyanins accumulation was detected. Berries collected at 58, 63, and 75 DAF represented the ripening phase, with intense sugar accumulation, malic acid breakdown, and secondary metabolite accumulation. The second growth phase physiologically ends with the maximum berry expansion (75 DAF) with the phloem influx arrest and the stop of a series of aquaporin channels and sugar transporters on the plasma membrane and tonoplast, together with many expansin genes (Savoi et al., 2021). Finally, berries at 88 DAF indicated the phase of shriveling, where principally sugars are not unloaded anymore but simply concentrated due to water transpiration. These eleven developmental stages were selected for a deep fruit transcriptomic study.

Before modeling, the genes were filtered by removing the low-expressed genes by keeping only the ones above 1 RPKM in at least one developmental phase (triplicate). A total of 14,869 genes were retained and tested for finding genes with significant temporal expression changes. After the statistical test, 4,514 genes were found non-significantly modulated overtime during the eleven phases of development. Consequently, the genes significantly modulated over the entire grape development and ripening cycle were 10,355. These genes were further grouped in clusters with similar expression patterns. For this analysis, an output of 25 clusters was defined (Fig. 1).

Most clusters (clusters 01-12 in Fig. 1) were allocated to the first green growth and lag phases, accounting for circa half of the considered genes (4,565). According to single berry phenotyping and transcripts cluster shape and height, the green period can be ultimately described with genes specifically switched on before malate and growth reached their maximal rate (clusters 01-05) and those specifically expressed when both expansion and malate accumulation reached maximum rates (clusters 06-12); in both cases, these green stage-specific genes were turned off before the beginning of ripening.

In the second growth phase, only 8 clusters (clusters 13-20 in Fig. 1) representing 2,037 genes were switched on during the critical phases of berry ripening. Also in this case, according to single berry phenotyping and transcripts cluster shape and height, the ripening period can be described with genes specifically switched on at softening and set off at the arrest of phloem (cluster 13-15) and genes highly expressed only during the late phase of ripening and shriveling period (cluster 16-20). All these genes were commonly not actively transcribed during the green period.

Lastly, five clusters (clusters 21-25 in Fig. 1), represented by 3,753 genes, showed a low degree of variation among phases, possibly indicating constitutive genes expressed in all phases of development.

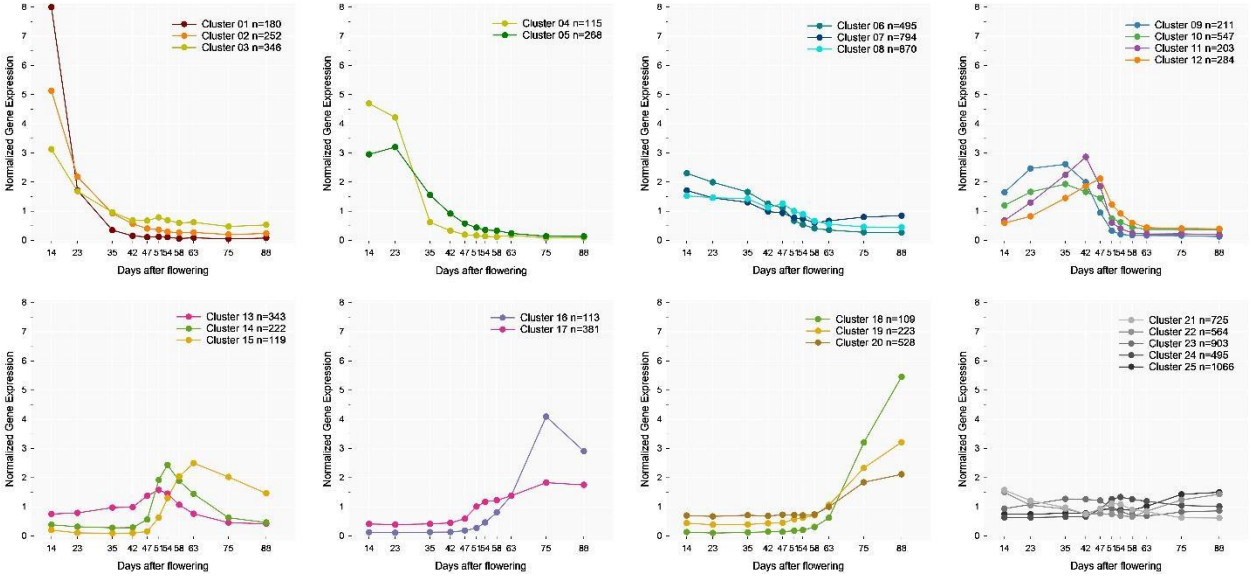

Figure 1. Genes modulated by developmental time identified by time-series analysis grouped in twenty-five clusters. The cluster number with the corresponding quantity of allocated genes is depicted within each graph. Softening was recorded 51 days after flowering (DAF), marking the separation between the green period (first phase of growth and lag phase) and the ripening period (second phase of growth and terminal shriveling).

4. Conclusions

Overall, the study provides new insights into the metabolic processes underlying the growth and ripening of grape berries and highlights the importance of considering individual berry development to understand better the heterogeneity observed within grape populations. Furthermore, phenotyping at single berry at fruit level can be an asset in better assessing the metabolic and water import fluxes. Finally, the regulation of grape ripening is a complex process involving multiple genes and pathways, and ongoing research is likely to deepen our understanding and identify additional genes and regulatory mechanisms involved in this important agricultural crop. Ultimately, some of this new knowledge may be translated to other fleshy fruit, generating results and networks of broad significance.

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6. Literature cited

Bigard A, Romieu C, Ojeda H, Torregrosa LJ-M (2022) The sugarless grape trait characterised by single berry phenotyping. OENO One **56**: 89–102

Coombe BG (1992) Research on development and ripening of the grape berry. Am J Enol Vitic **43**: 101–110

De Bei R, Wang X, Papagiannis L, Cocco M, O'Brien P, Zito M, Ouyang J, Fuentes S, Gilliham M, Tyerman S, et al (2019) Postveraison Leaf Removal Does Not Consistently Delay Ripening in Semillon and Shiraz in a Hot Australian Climate. Am J Enol Vitic **70**: 398–410

Gutiérrez-Gamboa G, Zheng W, Martínez de Toda F (2021) Current viticultural techniques to mitigate the effects of global warming on grape and wine quality: A comprehensive review. Food Res Int **139**: 109946

van Leeuwen C, Destrac-Irvine A, Dubernet M, Duchêne E, Gowdy M, Marguerit E, Pieri P, Parker A, De Rességuier L, Ollat N (2019) An update on the impact of climate change in viticulture and potential adaptations. Agronomy. doi: 10.3390/agronomy9090514

Nueda MJ, Tarazona S, Conesa A (2014) Next maSigPro: updating maSigPro bioconductor package for RNA-seq time series. Bioinformatics **30**: 2598–2602

Palliotti A, Frioni T, Tombesi S, Sabbatini P, Cruz-Castillo JG, Lanari V, Silvestroni O, Gatti M, Poni S (2017) Double-pruning grapevines as a management tool to delay berry ripening and control yield. Am J Enol Vitic **68**: 412–421

Rienth M, Torregrosa L., Ardisson M., De Marchi R., Romieu C. (2014) Versatile and efficient RNA extraction protocol for grapevine berry tissue, suited for next generation RNA sequencing. Aust J Grape Wine Res **20**: 247–254

Savoi S, Santiago A, Orduña L, Matus JT (2022) Transcriptomic and metabolomic integration as a resource in grapevine to study fruit metabolite quality traits. Front. Plant Sci. 13:

Savoi S, Torregrosa L, Romieu C (2021) Transcripts switched off at the stop of phloem unloading highlight the energy efficiency of sugar import in the ripening V. vinifera fruit. Hortic Res **8**: 1–15

Velt A, Frommer B, Blanc S, Holtgräwe D, Duchêne É, Dumas V, Grimplet J, Hugueney P, Lahaye M, Kim C, et al (2022) An improved reference of the grapevine genome supports reasserting the origin of the PN40024 highly-homozygous genotype. 2022.12.21.521434

OIV (2021) Annual Assessment of the World Vine and Wine Sector in 2021.