Single berry development - a new phenotyping and transcriptomics paradigm

Stefania Savoi, Laurent Torregrosa, Charles Romieu

Stefania Savoi[1,2]*, Laurent Torregrosa[3], Charles Romieu[1]*

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Affiliations
[1]UMR AGAP, Montpellier University, CIRAD, INRAe, Institut Agro-Montpellier, Montpellier, France
[2]Department of Agricultural, Forest and Food Sciences, University of Turin, Grugliasco, Italy
[3]UMR LEPSE, Montpellier University, CIRAD, INRAe, Institut Agro-Montpellier, Montpellier, France

Correspondence
Stefania Savoi*: stefania.savoi@unito.it, Laurent Torregrosa: laurent.torregrosa@supagro.fr, Charles Romieu: charles.romieu@inrae.fr

Summary

Most present knowledge on berry development has been obtained from a random sampling of hundreds of berries to average their diversity of the experimental plot. According to recent studies, such heterogeneous samples formed from non-synchronized berries of mixed developmental stages are unsuitable for detecting fast physiological and molecular changes. Thus, it is necessary to revisit the physiological and transcriptional bases of berry ripening. Here we report the in-depth study of the late-ripening program in three genotypes. Berry expansion during the second growth phase was characterized on-vine through image analysis. Hundreds of sampled berries were individually analyzed for primary metabolites to calculate their respective accumulation rates with high precision. These primary individual fluxes and the growth kinetics allowed us to distinguish targeted developmental stages further investigated through RNA profiling. Single berry monitoring evidenced sharp developmental phases during which specific genes or pathways are quickly switched ON or OFF. The comparison between Syrah and the two microvines showed phenotypic differences in late-ripening stages in vines grown in the field (Syrah) and microvines (MV032 and MV102) grown in the greenhouse. This study shows that new high-throughput single berry phenotyping methods are required to compare unambiguous developmental stages in physiological or genetic studies.

Key words

grapevine, berry ripening, image analysis, RNA-Sequencing, gene regulation

Introduction

The concept of single berry in grapevine physiological studies was first adopted by Coombe in the eighties of the past century (Coombe and Bishop, 1980; Coombe, 1980; Coombe and Phillips, 1982). Nowadays, it is an innovative strategy to study in-depth fruit development and its fine molecular processes. The foundations of this concept can be traced back to the observation that grape bunches ripen non-synchronously. Reports indicate that asynchronous berry bunch development is one of the primary reasons for the shifts in berry weight and composition at harvest among and within bunches of the same vineyard (Pagay and Cheng, 2010). This heterogeneity in the ripening status of berries has a direct link to the berry's mechanical properties or berry texture (Doumouya *et al.*, 2014). This likely new sorting criterion can be implemented by the wine industry for monitoring grape maturity and quality, for instance, to enhance secondary metabolites' skin extractability (Rolle *et al.*, 2012). The developmental heterogeneity is believed to begin before fruit set, most likely in the floral primordium differentiation at budburst, affecting vineyard yield and berry composition (Gray and Coombe, 2009). Classically, most grapevine transcriptomics studies randomly sampled the berries from one or multiple bunches, usually on the same day, and pulled the berries together in order to obtain one representative biological replicate, not considering at all the asynchrony delay in the sampling. Therefore, without reliable methods for guaranteeing synchronous populations of berries, Coombe (1992) was the first to suggest studying the development of grapes as a group of single berries, sequentially and non-destructively, while on the vine, by measuring berry diameters and softness or similarly sorted by firmness and color (Lund *et al.*, 2008).

This methodology was refined over the years by sampling and sorting the berries by density according to floatability in NaCl solutions (Bigard *et al.*, 2019; Carbonell-Bejerano *et al.*, 2016) rather than sucrose solutions (Nelson *et al.*, 1963; Singleton *et al.*, 1966). This reiterative selective process allows the separation and homogenization of the berries in specific developmental phases to obtain an equivalent set of fruits for physiological or omics comparisons. Furthermore, another way of sorting the berries was obtained thanks to the analysis in single berry of primary metabolites, such as organic acids and sugars, coupled with image analysis to increment the level of detail of the developmental time based on the values of the accumulation and degradation of such compounds and the berry growth (Shahood *et al.*, 2020). In this way, for the

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first time, single fruit kinetics of primary metabolites during berry ripening were extensively defined. Finally, the latest advancement was the addition of single fruit growth kinetics, calculated by volume changes based on frequent pictures, to the primary metabolites analysis and skin firmness and color change assessment coupled with transcriptomics (Savoi *et al.*, 2021) during the entire developmental cycle of berry ripening.

In general, this sampling strategy was successfully adopted in physiological studies (Bigard *et al.*, 2022, 2019; Shahood *et al.*, 2020; Hernández-Montes *et al.*, 2021), transcriptomics works aimed at studying abiotic responses of individual berries to temperature stress (Rienth *et al.*, 2014, 2016), biotic stress such as the leafroll virus infection (Ghaffari *et al.*, 2020), exogenous ozone application (Campayo *et al.*, 2021), and for understanding the arrest of phloem in ripening berries (Savoi *et al.*, 2021). Finally, DIGE and iTRAQ proteomics works of berry development and fruit quality traits were adopted to understand the relative contribution of pericarp/mesocarp tissues (Martínez-Esteso et al., 2011a, 2011b, 2013).

Here, we provide a three-genotypes comparison of single berry gene expression dataset highlighting common traits on primary metabolism, key berry physiological processes, and environmental-specific peculiarity in the fruit late-ripening program. New high-throughput technologies and upgraded data mining tools allowed us to observe and analyze fast changes in fruit ripening, especially at the phloem arrest (Savoi *et al.*, 2021), and here to understand distinctive differences among genotypes, especially between 'Syrah' and the two microvines, and their environments of cultivation.

Material and Methods

Plant materials and experimental layout

The study characterized three genotypes: the cultivar 'Syrah' (VIVC number: 11748), a broadly used variety in wine-making adapted to warm climates, and two hermaphroditic semi-dwarf microvines (originated from *Muscadinia rotundifolia* G52 and *V. vinifera* genotypes crossing) (Torregrosa *et al.*, 2019), named MV032 and MV102 as detailed in Savoi *et al.* (2021). These two varieties differed in carrying resistance genes; in fact, the *rpv1-run1* locus, conferring an elevated tolerance level to fungal diseases (Feechan *et al.*, 2013), was present in MV102, while absent in the susceptible microvine MV032. Furthermore, the 'Syrah' vines were cultivated in an open field at the experimental vineyard of Institut Agro Montpellier (France), whereas the two-year-old potted microvines were grown in a semi-controlled greenhouse with a range of 25°C and 15°C for day and night temperature respectively, a vapor pressure deficit of 1 kPa, photoperiod of 12 h of light per day.

The ripening of single berries was monitored through biweekly pictures of the same grape bunches (n=8), from softening (indicated with V for veraison), as in Savoi *et al.* (2021). Briefly, pictures were taken in the field or greenhouse between 9 and 11 am, using a Lumix FZ100 camera (Panasonic), maintaining the focal range and the distance from the bunch (30 cm) constant. The single berry volume of selected berries was calculated with the ImageJ software (Schneider *et al.*, 2012) by automatically counting the pixels within each targeted berry area, measured as an elliptical section. Each image was calibrated thanks to the 1-cm-scale present in each picture. The estimation of the berry volume was mathematically calculated using the radius of the targeted berry area. For observing the changes in volume during ripening, each single berry growth profile was normalised to its softening volume, set to 1. Based on these calculations, approximately 45 berries for each genotype were sampled at defined ripening stages (at mid-second growth – G; close to the berry maximum volume – P; during shriveling two weeks after the maximum volume – S). Seeds were removed, and the berries snap-frozen in liquid nitrogen in the field. Once in the laboratory, they were ground under liquid nitrogen operating with a ball mixer mill (Retsch MM400) in order to obtain a fine powder used in the following analyses.

Berry primary metabolites and transcript measurements

Single berries (skin + pulp together) were analyzed for sugar (glucose and fructose) and organic acids (malic and tartaric acids) by high-pressure liquid chromatography (HPLC), as detailed in Savoi *et al.* (2021).

By matching the growth kinetics data with the primary metabolites results, three individual berries per stage for each genotype were selected for RNA extraction. cDNA libraries were prepared with the Illumina TruSeq RNA Library Prep Kit v2. Samples were sequenced in paired-end mode, 2x150 bp reads, on an Illumina HiSeq3000 at the Genotoul platform of INRAe-Toulouse.

Raw reads were trimmed for length and quality, aligned against the reference grapevine genome PN40024 12X2, and counted with the latest Cost.V3 annotation, as detailed in Savoi *et al.* (2021).

Genes with less than 1 Read Per Kilobase per Million mapped reads (RPKM) were removed due to their low expression level. The resulting genes were tested for multi-time-series significance using the R package MaSigPro (Nueda *et al.*, 2014) with parameters degree=3, counts=T, rsq=0.8, k=8. One gene cluster was discharged (Supplementary File 1), as it pointed out genes non-modulated during the late-ripening stages under study. This gene cluster showed a high expression at softening (stage V), taken as a starting point in this expression analysis. These genes were not expressed further in the following stages of interest (G, P, and S).

Genes belonging to the same expression cluster or, in some cases, a couple of gene clusters with similar trends, were inferred for Gene Ontology (GO) functional enrichment analysis based on the available *Vitis vinifera* annotation, with the web tool g:Profiler using standard parameters (Raudvere *et al.*, 2019).

Results and Discussion

The successful sampling of synchronized berries from several grape bunches was achieved by applying non-destructive monitoring of single berry growth based on recurrent pictures of the same grape bunches during the ripening phase. In this way, berries still growing and importing water and photosynthates (G stage) were not mixed with ripe and over-ripe ones (stages P and S, respectively), in which these processes are stopped. We identified prevalent transcripts across different genotypes and environments that contribute to the accumulation during ripening of 1 M hexoses in this non-climacteric fleshy fruit (Savoi *et al.*, 2021). However, we further observed that genes unrelated to this essential physiological process were expressed with slightly different expression trends and/or magnitude in late-ripening stages between Syrah vines grown outside in an open field and the two microvines (MV032 and MV102) grown inside in a greenhouse.

The results of the transcriptomics analysis revealed significant modulation in gene expression profiles among the genotypes during ripening. The 5,037 genes modulated over time were grouped into seven gene clusters based on these differences (Fig. 1, Supplementary File 2). The GO inspection of these differentially enriched categories allows us to gain insights into the molecular mechanisms underlying the phenotypic differences in late-ripening stages due to GxE interactions. The gene set enrichment analysis mapped the clustered genes to known functional information sources for detecting statistically significant enriched terms (Fig. 2, Supplementary File 3).

Gene clusters 1 and 2, with 1,001 and 454 genes, respectively, were characterized by genes expressed during the early phase of berry ripening followed by a decreasing trend towards the end of the process, being switched off at the maximum berry expansion (stage P). The GO analysis revealed that the *carbohydrate metabolism process*, *water channel activity*, and *transporter activity* were the categories of interest represented in these gene clusters (Fig. 2, Supplementary File 3). In gene cluster 1, Syrah genes were expressed at a higher intensity than the two microvines (Fig. 1), while in gene cluster 2, this behavior was swapped. A striking difference between these two gene clusters is indicated by the expression intensity of some genes that lead, for example, to minor variations in fruit quality traits. The change in berry volume from veraison to the maximum berry expansion (V to P) measured in the three genotypes was not too divergent. In fact, Syrah berries recorded an increment from veraison to the maximum berry volume of + 70%, very similar to the change in volume during the ripening of MV102 (+69% increment); only for MV032, a higher berry volume increment of + 86% was recorded (Fig. 1a in Savoi *et al.*, 2021). However, a greater sugar accumulation was measured at the P stage in Syrah (1.2 M), indicated by a 35% higher sugar accumulation at phloem arrest than MV032 (0.8 M) and MV102 (0.7 M) (Fig 1b in Savoi *et al.*, 2021). This corresponded with a higher expression in field conditions of key sugar transporters such

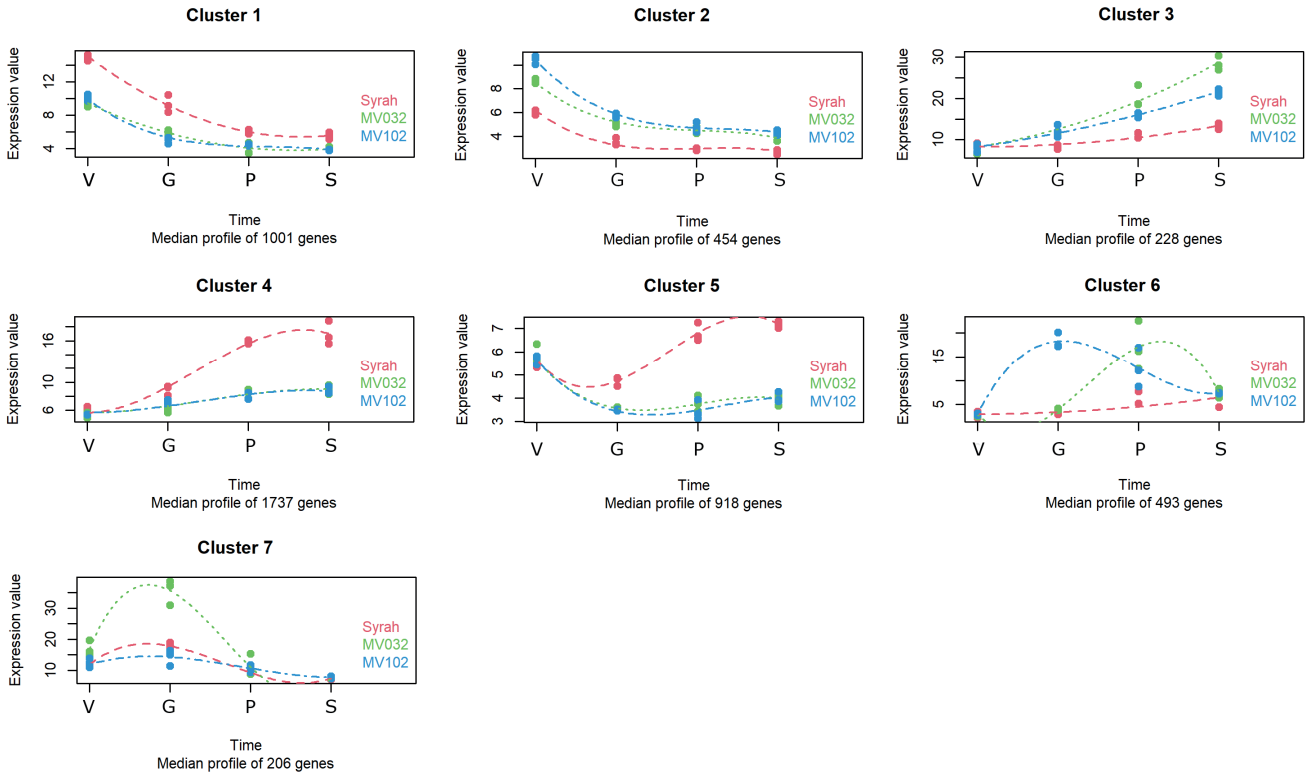

Fig. 1: Expression profiles of genes modulated over time subdivided into seven different gene clusters. Time on the x-axis is as follows: V as veraison – berry softening, G for growing ripening berries, P for berries close to their peak volume, and S for berry during the shriveling phase. Dashed lines represent the regression fit curves after MaSigPro analysis of each genotype through time. Red, green, and blue colors denote Syrah, MV032, and MV102 vines. The number of genes in each gene cluster is indicated below the corresponding graphs.

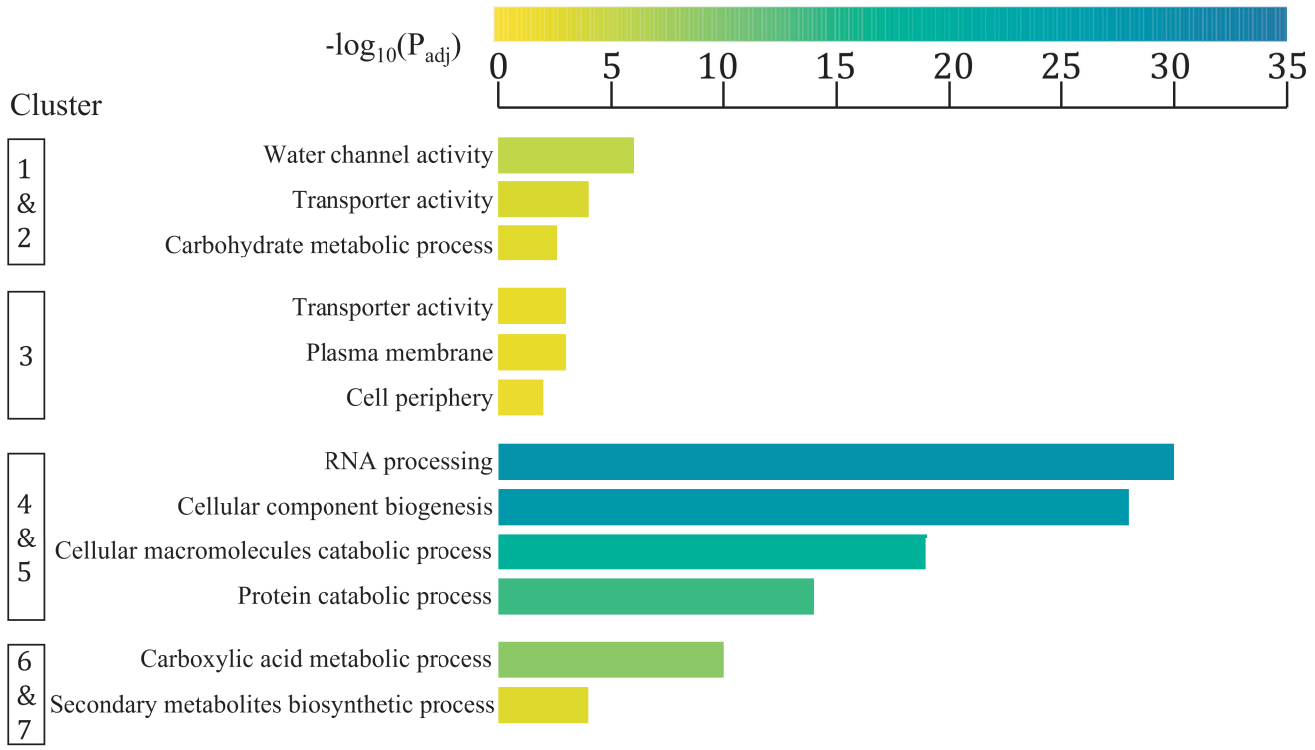

Fig. 2: A short list of terms enriched after the g: Profiler analysis is represented as colored bars for the gene clusters previously identified. Darker tones and longer bars denote a higher significance of the category mentioned on the left side, indicated as $-\log_{10}(p_{adj})$. The complete list is available in Supplementary File 3.

as *HT6* and *SWEET10* (gene cluster 1). Also the majority of aquaporins expressed in fruits (Wong *et al.*, 2018), such as *PIP1.3*, *PIP2.3*, *TIP1.2*, *TIP1.3*, etc., were higher expressed in field conditions (gene cluster 1), even though the growth was similar among genotypes. These results indicate a major environmental demand with higher osmotic pressure and water transpiration on 'Syrah' berry physiology. In external conditions, berries are exposed to direct sunlight and fluctuating temperatures during the day to a greater extent than in the greenhouse, requiring additional transcription of transporters genes in order to obtain a similar growth rate in a more stressful environment. Concerning the two microvines, these minor differences in sugar accumulation and berry expansion may be due to a certain level of berry plasticity (Dal Santo *et al.*, 2013) or hydraulic conductance traits associated with genetic diversity (Tardieu and Simonneau, 1998), which would be interesting to follow in more focused studies in well-watered versus water-deficit conditions.

In cluster 3, only 228 genes were allocated, showing an increased expression pattern trend from veraison until the over-ripening stages. However, the limited number of genes assigned to this cluster also impaired the GO analysis, indicating only a few enriched categories (Fig. 2, Supplementary File 3). Most of these genes were related to *transport activity*, and interestingly, *plasma membrane* and *cell periphery* were indicated as the enriched cell component categories. Among them, we noticed *SWEET15*, which was previously indicated as a constitutive sugar transporter as it is not repressed at the phloem stop but is pursuing its activities in ripe and over-ripe berries, most probably transporting sugar molecules between cell-to-cell, allowing an exchange from the core to periphery tissue (Savoi *et al.*, 2021). This agrees with the findings reporting *SWEET15* still active and actually up-regulated during postharvest dehydration, where this gene was measured as 3-fold higher in berries subjected to five days of dehydration (Conde *et al.*, 2018).

Clusters 4 and 5 represent those genes (1,737 and 918, respectively) highly modulated by the environmental conditions. The expression pattern indicated an interesting behavior that distinguished Syrah from the two microvines, *i.e.*, the field *versus* the greenhouse conditions. The GO analysis revealed the highest number of categories enriched as well as the most significant (Fig. 2 and Supplementary File 3). These genes were involved in transcription and translation processes and protein folding, sorting, and degradation, otherwise known as genetic information processing network components. Fasoli *et al.* (2018) previously identified this group of categories in a transcriptomics study as part of the late-ripening genes expressed with equivalent intensity in both 'Pinot noir' and 'Cabernet Sauvignon' varieties growth in an open field. This specific class of genes was further described as part of the third and final wave of gene expression during the development and ripening of berry fruit (Zenoni *et al.*, 2021). The distinction in gene magnitude expression between Syrah and the two microvines can be attributed to a higher protein turnover in field conditions. While environmental factors may influence the protein turnover rate in grapevine, the variability and control of these factors differ. The environment can be better controlled and managed in greenhouse conditions, leading to more stable protein turnover rates. On the contra-

ry, with a more erratic environment in the field, more variabilities in protein turnover rates can subsist. This machinery (enriched in these clusters), highly expressed in field conditions, works in both the synthesis and degradation of transcripts and proteins and indicates that protein turnover is a critical process for maintaining cell homeostasis. It enables the elimination of damaged proteins, recycling of their amino acids for the synthesis of new ones, and maintenance of protein levels, all of which contribute to the proper functioning of the cell without weakening crucial primary metabolic processes. In fact, among the most expressed genes, there was a *polyubiquitin*, which primary function is tagging proteins for proteasomal degradation, but also a *translation initiation factor eIF-1A*, one of the most critical regulatory factors of protein synthesis.

Finally, clusters 6 and 7, with 493 and 206 genes, respectively, empathized the microvine-specific genes as opposite to 'Syrah'. In particular, the category *secondary metabolic biosynthetic process* was indicated in the KEGG pathway results (Fig. 2 and Supplementary File 3). In fact, some phenylpropanoid and stilbene pathways genes were more expressed, such as several *phenylalanine ammonia-lyases*, and a conspicuous number of *stilbenes synthases* and their regulators *MYB14* and *MYB15* (Höll *et al.*, 2013). Currently, we do not have information on the pattern and intensity of accumulation of these secondary metabolites in these genotypes, as they were not measured. However, we can confirm that the berry clusters were visibly unaffected by diseases at the sampling time. Interestingly, by confronting the relative expression of the STSs (grouped in cluster 6) in the two microvines, the resistant MV102 showed a 100-fold higher expression in ripening growing berries, for then decreasing at ripe and over-ripe berries at a comparable level with MV032. The higher expression of stilbene genes hints toward a constitutive characteristic trait of this resistant hybrid. Further analyses coupling omics techniques will be helpful to characterize in deep MV102 and other resistant genotypes.

The ripening of fleshy fruits involves a complex transcriptomic reprogramming (Fasoli *et al.*, 2012) that leads to softening, accumulation of soluble sugars, synthesis of pigments, and development of aromatic compounds. As a non-climacteric fruit, grape ripens on the vine and accumulates sugars through phloem sucrose unloading (Zhang *et al.*, 2006). Many transcriptomic studies addressed grapevine physiology and berry development (reviewed in Savoi *et al.*, 2022), but only our work has focused on the inhibition of sugar phloem unloading at the end of the ripening process of berries and the transcriptomic modulation that triggers phloem switch-off on physiologically ripe berries (Savoi *et al.*, 2021). Sorting individual berries according to their sugar accumulation and water entry rates (measured as volume growth rate) helped us to elucidate the physiology of sugar accumulation in grapes. The mechanisms of sugar unloading in grapevines are now disclosed, suggesting that a sucrose/H^+ exchange at the tonoplast plays a central role in the energization of the fruit sink strength (Shahood *et al.*, 2020; Savoi *et al.*, 2021). This result was confirmed in different genotypes grown in an open field and in a greenhouse.

Conclusion

Single berry approaches have clearly shed new light on the physiological and molecular process of grape berry development and allowed the identification of genes that regulate phenotypic transitions, pathways, and metabolite accumulation. The precise information on the berry phenological status indicated by the measurement of four metabolites (glucose, fructose, malate, tartrate), together with knowledge of berry volume changes, has made it possible to distinguish individual berry phases for an unambiguous transcriptomic signature. The initial study focused on the inhibition of sugar phloem unloading during grape berry maturation and the transcriptomic modulation that triggers phloem switch-off. The results revealed prevalent transcripts that contribute to the accumulation of hexoses during ripening. This information was implemented by analyzing additional clusters of genes significantly modulated among genotypes during ripening. This study provides insights into the molecular mechanisms underlying the phenotypic differences in late-ripening stages in vines grown in the field and microvines in the greenhouse. The grapevine (and the microvines) is considered a model system for studying berry development and ripening of non-climacteric fleshy fruits. Overall, this study contributes to our understanding of the ripening process in grapes, and ongoing research will likely identify putative markers of fruit quality traits that could be of interest in breeding programs.

Conflicts of interest

The authors declare that they do not have any conflicts of interest.

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