



HAL
open science

Time-resolved transcriptomic of single *V. vinifera* fruits: membrane transports as switches of the double sigmoidal growth

Stefania Savoi, Mengyao Shi, Gautier Sarah, Audrey Weber, Laurent
Torregrosa, Charles Romieu

► To cite this version:

Stefania Savoi, Mengyao Shi, Gautier Sarah, Audrey Weber, Laurent Torregrosa, et al.. Time-resolved transcriptomic of single *V. vinifera* fruits: membrane transports as switches of the double sigmoidal growth. 2024. hal-04739970

HAL Id: hal-04739970

<https://hal.science/hal-04739970v1>

Preprint submitted on 16 Oct 2024

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

1 **Time-resolved transcriptomic of single *V. vinifera* fruits: membrane transports**
2 **as switches of the double sigmoidal growth**

3

4 Stefania Savoi^{1,2}, Mengyao Shi¹, Gautier Sarah¹, Audrey Weber¹, Laurent Torregrosa³,
5 Charles Romieu^{1*}

6

7 ¹UMR AGAP, Montpellier University, CIRAD, INRAe, Institut Agro, 34060, 2 Place Viala,
8 Montpellier, France.

9 ²Department of Agricultural, Forest and Food Sciences, University of Turin, Largo Braccini 2,
10 10095, Grugliasco, Italy.

11 ³UMR LEPSE, Montpellier University, CIRAD, INRAe, Institut Agro, 34060, 2 Place Viala,
12 Montpellier, France.

13 * corresponding author: charles.romieu@inrae.fr

14

15 **Email addresses of authors:**

16 stefania.savoi@unito.it

17 mengyao.shi@supagro.fr

18 gautier.sarah@inrae.fr

19 audrey.weber@inrae.fr

20 laurent.torregrosa@institut-agro.fr

21 charles.romieu@inrae.fr

22

23 **Date of Submission: 27/09/2024**

24 **Number of Figures: 7**

25 **Number of Tables: 0**

26 **Supplementary Data:** 1 supplementary figure, 7 supplementary tables

27

28 **Word Count: 6853**

29 Introduction: 986

30 Results: 3671

31 Discussion, Conclusion: 2196

32

33 **Running title:** Transcriptomic clock in a non-climacteric fleshy fruit

34

35

36

37 **Highlights**

38 To alleviate asynchronicity biases, transcripts showing strict coincidental timing with pericarp
39 physiological phases were disentangled on single berries, enlightening the tight multifaceted
40 membrane developmental control of sugar and acid fluxes.

41

42 **Abstract**

43 By revealing that the grape berry loses one H⁺ per accumulated sucrose at the inception of
44 ripening, adopting a single fruit paradigm elucidates the fundamentals of the malate-sugar
45 nexus, previously obscured by asynchrony in population-based models of ripening. More
46 broadly, the development of the individual fruit was revisited from scratch to capture the
47 simultaneous changes in gene expression and metabolic fluxes in a kinetically relevant way
48 from flowering to overripening. Dynamics in water, tartrate, malate, hexoses, and K⁺ fluxes
49 obtained by combining individual single fruit growth and concentration data allowed to define
50 eleven sub-phases in fruit development, which distributed on a rigorous curve in RNAseq
51 PCA. WGCNA achieved unprecedented time resolutions in exploring transcript level-
52 metabolic rate associations. A comprehensive set of membrane transporters was found
53 specifically expressed during the first growth phase related to vacuolar over-acidification.
54 Unlike in slightly more acidic citrus, H⁺ V-PPase transcripts were predominantly expressed,
55 followed by V-ATPase and PH5, clarifying the thermodynamic limit beyond which
56 replacement by the PH1/PH5 complex turns compulsory. Puzzlingly, *bona fide* ALMT kept a
57 low profile at this stage, possibly replaced by a predominating uncharacterized anion
58 channel. Then, the switch role of HT6 in sugar accumulation was confirmed,
59 electroneutralized by malate vacuolar leakage and H⁺ pumps activation.

60

61 **Keywords**

62 Energy metabolism, Fruit development, Grapevine, Organic acids, Proton pumps, Ripening

63

64

65

66

67

68

69

70

71

72

73 Introduction

74 Developing an edible pericarp to promote the dispersal of mature seeds displays convergent
75 features in Angiosperms. Before coloring and accumulating quick carbohydrates during
76 ripening to attract and provide frugivores an energy reward, green fleshy acidic fruits are
77 protected by astringent proanthocyanidins and undergo an initial expansion period relying on
78 the storage of organic acids as major osmoticums in their hypertrophied vacuoles (Batista-
79 Silva *et al.*, 2018; Rauf *et al.*, 2019). Whenever as indicated by its name, the major
80 accumulated organic acids may change within species, a similar acid trap mechanism
81 operates, based on keeping the vacuolar pH (pH_v) below the pK of the organic acid so that
82 Henderson-Hasselbalch equation forces transported anions to recombine with H⁺ in the
83 vacuolar lumen, thereby alleviating membrane depolarization, reducing pH_v decrease, and
84 subsequent transport retro inhibition (Martinoia *et al.*, 2007). This obvious lack of charge
85 neutralization by K⁺ also prevents the depletion of this mineral in the rhizosphere of a
86 perennial plant amenable to decades of fruit dissemination. Nevertheless, reaching an
87 extreme pH_v raises a bioenergetic challenge that is solved by recruiting a low stoichiometric
88 ratio P-type ATPase complex in lemon varieties with a pH_v of about 2.5 (Strazzer *et al.*,
89 2019). Not only should H⁺ V-ATPase and H⁺ V-PPase be unable to reach such pH_v beyond
90 their expected thermodynamic limits, but they would even collapse it by functioning in the
91 reverse mode, which is prevented by the unique conformation of the inhibitory H subunit in
92 citrus V-ATPase (Tan *et al.*, 2022). The predominant PH1/PH5 complex in citrus is
93 transactivated by the basic helix-loop-helix (bHLH) transcription factor *Noemi* (Butelli *et al.*,
94 2019), which in grapevine controls the possibly unrelated processes of acids and
95 proanthocyanidins accumulation upon acting in an MYB/bHLH/WD40 (MBW) complex,
96 whose orthologs in grapevine are represented by the bHLH *VviMYC1* (Hichri *et al.*, 2010),
97 the TFs *VviMYB5b* and *VviWRKY26* (Amato *et al.*, 2019). The PH5 ortholog is apparently
98 expressed in grapevine, but unlike in citrus, proteomic, enzymatic, or vectorial evidence
99 show that functional V-ATPase and V-PPase largely predominate in green grape berries at
100 pH_v 2.7 (Terrier *et al.*, 1998; Rienth *et al.*, 2016; Kuang *et al.*, 2019). Furthermore,
101 aluminum-activated malate transporters (ALMT), such as the apple *ALMT9*, or *Ma1* gene
102 (Bai *et al.*, 2012), play a critical role in fruit acidification upon transferring malate inside the
103 vacuole. Surprisingly, despite displaying suitable inward rectifying transport of malate and
104 tartrate, the gene described as *VviALMT9*, is preferentially expressed during the ripening
105 period rather than during the acid accumulation phase (De Angeli *et al.*, 2013), which
106 questions its practical implication in building grape acidity. A better temporary resolution
107 would shed light on a situation that is all the more puzzling as the quite uncommon tartaric
108 acid, stronger than malic acid, accumulates simultaneously with proanthocyanidins in the

109 very young berry. It is becoming increasingly clear that such energy accumulated as an H⁺
110 gradient will play a key role during ripening. Critical re-evaluations of sugar accumulation
111 versus malate breakdown at this stage led to observing an initial 2 hexoses/H⁺ stoichiometry
112 in all *vinifera* varieties investigated so far (Shahood *et al.*, 2020; Bigard *et al.*, 2022), in line
113 with the induction of the *VviHT6* transcript and protein that has been widely confirmed
114 (Kuang *et al.*, 2019; Savoi *et al.*, 2021) since the initial report of Terrier *et al.* (2005). This
115 raises the question of the exit route of malate before H⁺ pumping back by V-ATPase and V-
116 PPase takes over the electro-neutralization process, as evidenced on tonoplast vesicles
117 (Terrier *et al.*, 2001).

118 Grapevine (*Vitis vinifera*) is a major fruit crop and a model for non-climacteric fruits. The
119 latest process-based modeling studies are rooted in the crude approximation that berry
120 development would reflect the compositional changes of the future harvest as an average
121 population and reciprocally (Zhu *et al.*, 2019; Tornielli *et al.*, 2023). Individual berry
122 approaches show that current models of berry development are biased by asynchronous
123 ripening within the future harvest (Bigard *et al.*, 2019; Shahood *et al.*, 2020; Daviet *et al.*,
124 2023). Indeed, asynchronicity lies within the duration of phenological stages under study,
125 making average kinetics developmentally chimeric. Consequently, single-fruit
126 transcriptomics yielded a straightforward identification of genes suddenly switched off with
127 sugar accumulation at the end of ripening and provided decisive insights into the structure
128 and energetics of the phloem unloading pathway (Savoi *et al.*, 2021). Such a proof of
129 concept challenges us to revisit the whole berry developmental cycle from the strict kinetic
130 and quantitative point of view.

131 Recently, several studies have been dedicated to deciphering the signaling cascade
132 triggering the onset of ripening, a pivotal developmental point in fruit physiology. A quite
133 consensual hormonal pattern emerged, but which family or transcription factor members are
134 responsible for the transcriptomics reprogramming is still being determined. In non-
135 climacteric fruits, a slight increase of endogenous ethylene hormone is followed by a larger
136 peak of abscisic acid onsetting the veraison, prevented by auxins (as reviewed in Perotti *et al.*,
137 2023; Zenoni *et al.*, 2023). On the other hand, a whole range of NACs (D'Inca *et al.*,
138 2023), GRAS (Neves *et al.*, 2023), bHLH, WRKY (Fasoli *et al.*, 2018), LOB (Grimplet *et al.*,
139 2017) transcription factor families and two ripening time control (RTC1 and RTC2) proteins
140 (Theine *et al.*, 2021) would trigger ripening molecular events, without defining a conclusive
141 ripening cascade. The TF *VviERF27* was recently identified as the single candidate in the
142 veraison locus *Ver1* (Frenzke *et al.*, 2024), influencing ripening start. In this respect, results
143 from these disparate studies need to be synchronized on a common time frame relying on
144 unambiguous samples and internal clock markers.

145 Here, we report the first single fruit transcriptome study encompassing the complete three-
146 month period from anthesis to over-ripening in the world-cultivated Syrah variety. This new
147 approach yielded a straightforward identification of key transporters and bioenergetic players
148 switching abrupt metabolic changes during pericarp development. Ultimately, we attempted
149 to individuate the expression profiles of pivotal master regulators of the berry ripening
150 process.

151

152 **Materials and Methods**

153 **Plants**

154 The *Vitis vinifera* variety Syrah, an iconic variety for the production of red wines in regions
155 under temperate climates, was considered for this study in 2018 and 2019. The vines,
156 grafted on SO4 and planted in 2000 in the experimental facility of Institut Agro Montpellier,
157 were irrigated daily during the summer to avoid severe water shortage. Classical
158 phytosanitary treatments were applied over the season to ensure healthy plant and fruit
159 development.

160

161 **Single berry sampling**

162 The double sigmoidal growth pattern of single berries was monitored through recurrent
163 pictures of selected clusters on different plants, starting at flowering and continuing until two
164 weeks after maximum berry volume. Photographs were taken using a Lumix FZ100
165 (Panasonic), keeping the focal range and cluster to camera lens distance (30cm) constant.
166 The volumetric growth of single berries was calculated following picture analysis with the
167 ImageJ software. After calibrating all images with their 1 cm internal standard, berry edges
168 were manually adjusted to ellipsoids whose areas were determined by pixel counts before
169 estimating the berry volume as previously described (Savoi *et al.*, 2021).

170 The complete developmental period was followed for twenty berries, allowing to model their
171 individual double sigmoidal growth patterns, while a second set of 219 berries were sampled
172 according to their own volume changes over two seasons (2018 and 2019) before sugar,
173 organic acids, and K⁺ were analyzed on circa 0.1 g aliquots of frozen samples, as described
174 below. Calendar dates were converted to individual DAF ones, following resynchronization
175 based on berry growth, composition, and softening dates. The net fluxes of water (growth),
176 organic acids, and sugar accumulation were calculated for each berry before selecting the
177 eleven time points for RNAseq samples (Table S1). To avoid circadian cycle influences
178 (Rienth *et al.*, 2014; Davies *et al.*, 2023), pictures and berries were sampled at the same
179 time of the day, between 9 and 11 AM. Berries without pedicel were rapidly deseeded before
180 freezing in liquid N₂ (within 1 min after harvest) and stored at -80°C. A total of 219 single

181 berries were ground to a fine powder under liquid N₂ using a stainless steel ball mill (Retsch
182 MM400).

183

184 **Primary metabolites analysis**

185 Each single berry was analyzed for sugars (glucose and fructose) and acids (malic and
186 tartaric acids) by high-performance liquid chromatography. For each 219 berries, 100 mg of
187 frozen powder was diluted 6x with a solution of HCl 0.25 N, well-shacked, and left overnight
188 at room temperature. Samples were then centrifuged at 13,000 g for 10 min, and a
189 supernatant aliquot was diluted 10x with a solution of H₂SO₄ 5mM containing 600 μM of
190 acetic acid as internal standard. Samples were transferred to HPLC vials and injected,
191 according to Rienth *et al.* (2016). For K⁺ analysis, the previous samples in HCl 0.25 N were
192 further 10x diluted with MilliQ water and analyzed with atomic absorption spectroscopy as in
193 Bigard *et al.* (2020). Data were expressed as mEq for organic acids or mM for sugar
194 concentration; content per fruit (concentration x volume) was defined as mEq/Nfruit or
195 mM/Nfruit where N represents the number of fruits needed to reach 1 kg FW at maximal
196 volume, which was 485 for Syrah.

197

198 **RNA extraction and sequencing**

199 To resynchronize berries at homogeneous developmental stages, single berry triplicates
200 (pericarp) were selected based on relative growth, sugars, and organic acids, yielding 33
201 samples before individual RNA extraction and library preparation as in Rienth *et al.* (2016).
202 Samples were sequenced on an NGC Illumina HiSeq3000 in paired-end mode, 2x150 bp
203 reads, at the Genotoul platform of INRAe-Toulouse.

204

205 **Transcriptomics data analysis**

206 Raw reads were trimmed for quality and length with “Trimmomatic”, version 0.38 (Bolger *et al.*
207 *et al.*, 2014). Reads were aligned against the reference grapevine genome PN40024 12X2
208 (Canaguier *et al.*, 2017) using the software “Hisat2”, version 2.1.0 (Kim *et al.*, 2015), yielding
209 an average of 31.6 M sequences per sample. Aligned reads were counted with “HTSeq-
210 count” (version 0.9.1 (Anders *et al.*, 2015)) using the VCost.v3 annotation. Genes were
211 filtered by applying an RPKM>10 cut-off in at least one experimental condition, resulting in
212 7266 expressed genes. Transcripts expressed as normalized RPKM were tested for multi-
213 time-series significance to screen those genes showing significant temporal expression
214 changes using the “MaSigPro” R package (Nueda *et al.*, 2014) with parameters degree=7,
215 rsq=0.7. The output, consisting of a list of 6374 time-modulated genes, accompanied by
216 osmolytes metabolic rates and phenological stages, was subjected to a weighted correlation
217 gene co-expression network analysis with the “WGCNA” R package (Langfelder and

218 Horvath, 2008) using the RPKM dataset, with parameters β power 30, network type signed,
219 min module size 100, deepSplit 1, MEDissThreshold 0.1. WGCNA resulted in the 13
220 following modules: black (560 genes), blue (660), brown (793), green (952), yellowgreen
221 (296), lightcyan (103), magenta (648), midnightblue (113), pink (348), tan (266), turquoise
222 (693), yellow (560), and finally grey (382) containing genes unassigned.

223

224 **Calculation of the transcriptomics clock**

225 A PCA was conducted on unfiltered variance stabilized transformed (VST, DESEQ) gene
226 expression data from the 33 sequenced samples. The transcriptomic time was calculated as
227 the curvilinear distance between the orthogonal projections of successive individual berry
228 data on a fitted polynomial line in the first PCA plane. The curvilinear distance was
229 approximated as the integral of $dl = R(\varphi).d\varphi$ following a change from cartesian to polar
230 coordinates. The rate of accumulation or degradation for each osmolyte was then retrieved
231 based on the transcriptomic time and the metabolic data.

232

233 **Results**

234 **Single fruit monitoring provides new kinetic bases for berry development**

235 Twenty individual berries were photographed every two to three days for three months,
236 starting a few days after flowering (DAF). Image analysis led to characterize typical double
237 sigmoidal growth curves followed by two weeks of berry shriveling, as accepted in the
238 grapevine, except for the facts that the herbaceous plateau appeared particularly long with a
239 continuous slow berry volume increment and the second intense growth period lasted 3.5
240 weeks, as previously highlighted on individual berries (Shahood *et al.*, 2020). Best fits with
241 the 4-parameter sigmoidal function $v=v_0+v_{max}/(1+\exp((t_0-t)/b))$ were obtained for each growth
242 phase, where v stands for fruit volume, t for time (days), v_0 and v_{max} initial volume and
243 plateau volume, t_0 the time at which both mid-growth and maximal growth rate were reached
244 (Fig. 1A and Table S2, S3). Asynchronicity was largely eliminated upon resynchronizing t_0
245 values, shifting from calendar time to single fruit-specific DAF. Average values of 23.14 and
246 62.05 DAF were obtained for t_0 green and t_0 ripening (i.e., for mid-first and mid-second
247 intense growth phases, respectively), resulting in an offset of 39 days. Following the
248 normalization of individual berry peak volumes, the first (or green) growth period plateaued
249 at 0.44 (Fig. 1A, Table S3), indicating a greater expansion during the second growth period
250 (ripening). Moreover, according to their respective maximal growth rates, the ripening berry
251 expands 1.74 times faster than during the green stage. Terminal shriveling was more or less
252 pronounced on different berries. On average, the berry volume decreased by 17% in three
253 weeks after the stop of phloem unloading, with a minimum decrease of 7% and a maximum

254 of 29% (Fig. 1A, Table S2). This decrease was not correlated to the berry surface-to-volume
255 ratio (not shown). On these 20 berries and those destructively sampled, the first intense
256 growth phase lasted until 35 ± 1.5 DAF on average, and softening (onset of ripening)
257 occurred at 51 ± 1.5 DAF. Berry skin coloration followed softening by about one week,
258 starting at about 58 ± 1.5 DAF, quite simultaneously with the second intense growth phase.
259 Maximum berry volume was reached at 75 ± 1.5 DAF, 24 days after softening (Table S1).

260

261 **Acids storage and breakdown during fruit development**

262 Tartaric acid, already present at 5 mEq/Nfruits at 9 DAF (Table S4), the first sampling date,
263 reached circa 87 mEq/Nfruits at 35 DAF (Fig. 1B) and remained stable after that, regardless
264 of a slight inflection at 47 DAF (the first sampling date of the second experimental season),
265 possibly due to different environmental conditions during the first growth phase (Becker *et al.*,
266 2022; Reluy *et al.*, 2022), even if data from Melino *et al.* (2009) showed the same
267 inflection at softening in a non-synchronized population of berries. Tartaric acid was still at
268 91 mEq/Nfruits at peak volume (75 DAF), which confirms it is only accumulated during the
269 first growth phase and not further metabolized during ripening. By contrast, malic acid, being
270 only 0.7 mEq/Nfruits at 9 DAF (Table S4), started to accumulate a few days later than
271 tartaric acid but continued until reaching a malate/tartrate ratio above 2 (151 mEq/Nfruits) at
272 circa 47 DAF (Fig. 1B). Malic acid was roughly constant around softening (51 DAF) and
273 suddenly dropped at about 58 DAF, displaying a pseudo-first-order decrease (Fig. 1D) until
274 reaching values of 41 mEq/Nfruits at 88 DAF. Our data distinctly states that the organic acid
275 present in the berry pericarp at our first metabolic sampling was tartrate, which anticipated
276 malate accumulation by a few days (Fig. 1B). Then, malate became 2-3 fold higher than
277 tartrate during the first growth period before ripening was triggered. The net accumulation
278 rates of tartaric and malic acid, the major osmoticums in the first water accumulation period,
279 were estimated for the first time on a per-fruit basis, exhibiting respective values of 3 and 4.4
280 $\mu\text{Eq}/\text{min.NFruits}$ when reaching a quasi-constant accumulation rate, as can be seen in Fig.
281 1B.

282 Potassium slowly accumulated during the green and lag stages (from 1 to 16 mEq/Nfruits),
283 accelerated during the ripening phase before reaching 59 mEq/Nfruits (Fig. 1B, Table S4) at
284 phloem arrest (Savoi *et al.*, 2021). Puzzlingly, K^+ accumulation was relatively insignificant
285 during the malate breakdown period accelerating afterward (Fig. S1).

286 During the green stage, the elevated glucose/fructose ratio above 3 (Fig. 1C) illustrates the
287 preferential use of fructose from imported sucrose as a carbon and energy source. However,
288 fructose consumed in excess of glucose should just allow the synthesis of 60% accumulated
289 malic acid, implying that a supplementary part of imported sucrose is entirely metabolized as

290 organic acids or pyruvate as a respiratory substrate. Actually, photosynthesis would never
291 reach the compensation point in berries (Niimi and Torikata, 1979).

292

293 **The sequence of events at the onset of ripening and sugar accumulation**

294 Sequential destructive analysis of single berries confirmed that the first phenotyping signs of
295 ripening were simultaneous softening and decrease of the G/F ratio below 3 (Fig. 1C),
296 subsequent to the marked acceleration of sucrose import and hydrolysis above 0.15 M
297 hexoses, while berry expansion resumed later, at 0.4 M hexoses. The combination of growth
298 and concentration data showed that at softening (51 DAF), glucose and fructose
299 accumulation progressively accelerated up to 63.7 $\mu\text{mol hexose}\cdot\text{min}^{-1}\cdot\text{Nberry}^{-1}$,
300 representing a 20-fold acceleration compared to the green stage (3.1 $\mu\text{mol hexose}\cdot\text{min}^{-1}\cdot\text{Nberry}^{-1}$),
301 before abruptly stopping 24 days later. However, the initiation of malic acid
302 breakdown did not occur before 0.3 M sugar (Fig. 1D). Once induced, the initial rate of
303 malate breakdown was in line with a 1 sucrose/1 H^+ exchange at the tonoplast (Fig. S1).
304 Finally, it is important to note that K^+ accumulated at a 25x lower rate than sugars during the
305 ripening phase.

306

307 **Transcriptomic overview**

308 For RNA sequencing, thirty-three berries representing eleven sub-phases of berry
309 development were selected among the 219 sampled ones, according to their individual
310 growth rate and primary metabolite contents. The resynchronized DAF of the triplicates of
311 single berry and their developmental and phenological acronyms are detailed in Table S1
312 and visualized in Fig. 2A. 54% and 15% of the variance in gene expression were distributed
313 on the two first axes of the principal component analysis (PCA) (Fig. 2B). The three single
314 fruits remained largely indistinguishable inside biological replicates, even if slight
315 divergences appeared at the R9 stage and beyond. The developmental stage clearly drove
316 sample distribution. Berries did not depart from the same line, illustrating the higher accuracy
317 of the transcriptomic clock compared to multi-trait phenotyping to synchronize berries. As
318 much as 14% of the top 100 influencing genes driving sample distribution on the negative
319 side of PC1 (from G1 to L5) encoded photosynthesis-related functions (Table S5).
320 Noticeably, one-half of the 100 most influencing transcripts on the PC1 positive side (from
321 S6 to Sh11) have been previously identified as “switch” genes (Palumbo *et al.*, 2014)
322 transcriptionally induced at softening and significantly activated during ripening. Therefore,
323 PC1 sample separation confirms the general transcriptomics reprogramming during the
324 ripening process (Fasoli *et al.*, 2012), clearly separating the first growth phase (including the
325 green plateau) from the ripening second one. Interestingly, the genes placing samples in the
326 negative section of PC2 (i.e., G1, G2, partially R9, R10, and Sh11) (Table S5) were more

327 particularly devoted to the phenylpropanoid and flavonoid pathways (but not anthocyanin
328 specific, which were listed in PC1+). By contrast, positive gene loadings for PC2 did not
329 belong to a particular class or known pathway.

330

331 **The single-berry transcriptomic clock**

332 The curvilinear distance on the fitted line passing through the 2D PCA of single-berry
333 transcriptomes was found to be roughly proportional to their resynchronized internal time,
334 which still suffers from uncertainties owing to the complexity of berry resynchronization
335 based on individual growth curves and primary metabolites (Fig. 2C). A significant
336 acceleration of transcriptomic changes occurred around softening, while homeostasis was
337 reached at maximum volume or phloem arrest. On individual berries, this approach,
338 conceptually inherited from Torielli *et al.* (2023), virtually eliminated the random noise on
339 uncharacterized, thus variable mixes of unsynchronized berries. Indeed, single berry
340 transcriptomic time (Table S6) obtained blindly from unfiltered RNAseq results allows dating
341 individual berries with a precision within the few days range, if not less, and outperforms all
342 alternative resynchronization procedures, as illustrated on post-veraison samples.

343

344 **Weighed gene co-expression network analysis**

345 After filtering for low-expressed genes, 6374 genes were significantly modulated over time.
346 Their subdivision into 12 different modules of clusters of highly correlated genes (Fig. 3A,
347 3B, Table S7) (disregarding the grey module that includes all unassigned profiles) allowed
348 us to study the transcriptomic regulation of metabolic fluxes and related traits with
349 unprecedented time precision.

350 No specific gene expression displayed a significant association with the berry growth rate on
351 both phases of the double sigmoidal curve (Fig. 3C). Examining the two growth phases
352 separately led to the identification of the lightcyan and midnightblue modules as highly
353 correlated (0.91 and 0.95) with the first growth phase, followed by brown, green, and tan
354 modules (0.76, 0.62, 0.61), (Fig. 3C). Modules black, brown, yellowgreen, and tan correlated
355 with the rate of tartaric acid (0.94, 0.88, 0.73, and 0.87) and malic acid synthesis (0.89, 0.76,
356 0.77, and 0.70), and the green phase in general, with some differentiation among G1 and G2
357 (Fig. 3C, 3D). Contrary to the growth rate, separating malic acid accumulation in the green
358 phase from its degradation during ripening, did not improve gene association with the net
359 accumulation rate (either positive or negative) during the complete cycle. The lag phase and
360 then L4 and L5 separately were associated with yellowgreen (0.73 for the lag, 0.75 for L4)
361 and pink (0.73 for the lag and 0.63 for L5) modules. The second growth rate was associated
362 with the yellow module (0.64) and the magenta (0.45) to a lesser extent. Similarly, the yellow
363 module correlated with the accumulation rate of sugar (0.68), softening (0.77), S6 (0.47),

364 and S7 (0.57). None of the modules showed a high correlation with the rate of K⁺ storage.
365 However, when the K⁺ accumulation rate was considered separately during the green-lag
366 and then softening-ripening phases, we could notice that modules brown, black, tan,
367 midnightblue, lightcyan, and greenyellow correlated with the berry first phase of growth
368 behavior, while yellow and magenta correlated with the ripening one. The ripening phase
369 was associated with the magenta module (0.63), while the terminal shriveling was with the
370 blue (0.57, and R10 with 0.71) and turquoise (0.64, and Sh11 with 0.84) ones. At the same
371 time, sugar concentration, but not accumulation rate, was strongly associated with the blue
372 (0.96), turquoise (0.89), and magenta (0.83) modules, as for K⁺ (0.93, 0.89, 0.83).

373

374 **Transcriptomic regulation of water fluxes and cell expansion**

375 Different subsets of aquaporins on the plasma or the tonoplast membranes and cell wall-
376 related genes were specifically expressed during each growth phase, both associated with
377 an eightfold expansion of the average cellular volume (Ojeda *et al.*, 1999). In particular, the
378 PIPs, TIPs, and EXPs expressed during the green phase were found in the black, tan,
379 brown, and green modules, while the ones activated at softening belonged to the yellow
380 module only; the two EXPs up-regulated later during ripening were grouped in the magenta
381 module instead. Moreover, genes of the same family were expressed at different
382 magnitudes, enlightening different genetic controls over these genes.

383 Specifically, two PIPs (*VviPIP2.7 - Vitvi03g00155* and *VviPIP2.4 - Vitvi06g00281*) and two
384 TIPs (*VviTIP2.1 - Vitvi09g00329* and *VviTIP1.1 - Vitvi06g01346*) exhibited an early green
385 stage-specific expression, as majorly emphasized by *VviPIP2.7* and *VviTIP2.1*, thus allowing
386 a continuous influx of water in green expanding berries, while showing a fast decrease
387 during the lag phase (Fig. 4A, 4B). Then, at softening (51 DAF), these 'green' isogenes were
388 mainly replaced by the expression of *VviPIP2.5 (Vitvi13g00605)* and *VviPIP2.3*
389 (*Vitvi08g01038*) on the plasma membrane and by *VviTIP1.2 (Vitvi08g01602)* and to a lesser
390 extent *VviTIP1.3 (Vitvi13g00255)* on the tonoplast (Fig. 4A, 4B). Furthermore, *VviPIP1.3*
391 (*Vitvi02g00310*) and *VviPIP1.4 (Vitvi15g01110)* were expressed during both phases of berry
392 growth until being repressed at the phloem arrest, stopping the influx of water into the berry.

393 *VviEXPA16 (Vitvi14g01977)* was the most expressed expansin gene during the first growth
394 phase and was slightly enhanced in the second one as well. A rather low RPKM, *VviEXPB02*
395 (*Vitvi12g00342*), was expressed only in the first growth phase. Three other isogenes were
396 abruptly switched on at softening, with *VviEXPA14 (Vitvi13g00172)* and *VviEXP19*
397 (*Vitvi18g00189*) showing the highest expression at the end of the lag phase; a similar trend
398 was observed for the less expressed *VviEXP18 (Vitvi17g01251)*. These genes were
399 progressively replaced by two other genes (*VviEXPA01 - Vitvi01g01030* - and *VviEXPB04 -*
400 *Vitvi15g00643*) induced during the second growth phase and clearly peaking with berry

401 growth rate at 58 DAF, still substantially expressed during over-ripening, indicating a role in
402 the turn-over of cell wall structure during the shriveling period (Fig. 4C, 4D).

403

404 **Proanthocyanidins and organic acid**

405 Five genes committed to the proanthocyanidins biosynthetic pathway or its regulation were
406 specifically expressed at the very beginning of berry development (green module), notably
407 leucoanthocyanidin reductase 1 - *VviLAR1* (*Vitvi01g00234*), anthocyanidin reductase -
408 *VviANR* (*Vitvi10g02185*), the PA transporters *VviPA-MATE1* (*Vitvi12g00101*) and *VviPA-*
409 *MATE2* (*Vitvi12g00099*), the TF *VviMYBPA2* (*Vitvi11g00099*) (Terrier *et al.*, 2009; Pérez-
410 Díaz *et al.*, 2014). However, both *VviMYBPA1* (*Vitvi15g00938*) and *LAR2* (*Vitvi17g00371*)
411 were clearly delayed (brown module) (Fig. 5A, 5B). The anthocyanins *VviMYBA1*
412 (*Vitvi02g01019*), *VviMYBA2* (*Vitvi02g01015*), their target gene *VviUFGT* (*Vitvi16g00156*),
413 and the anthocyanin MATE transporter (*Vvi-A-MATE*, *Vitvi16g01913*) showed a
414 simultaneous increase in expression a few days after softening (54 DAF, magenta module).

415 One of the most expressed genes (in RPKM) at 14 DAF was *VviVTC2* (GDP-mannose 3,5-
416 epimerase 1, *Vitvi19g00549*), the regulatory step in the ascorbate pathway in plants, as the
417 precursor for tartrate in grape, which exhibited a green stage-specific expression pattern
418 (Fig. 5C, 5D). The expression of *VviL-IDH3* (L-idonate hydrogenase, *Vitvi16g01858*), the
419 rate-limiting enzyme of tartrate synthesis, was also maximal at 14 DAF, decreasing in the
420 green stage until virtually annihilating during the sugar accumulation process, as in DeBolt *et*
421 *al.* (2006). Unexpectedly, this gene was reactivated in shriveling berries, contrary to
422 *VviVTC2*. These genes were placed in the brown and green modules, showing a greater
423 correlation with tartaric acid and stage G1. A far less documented, multifunctional enzyme in
424 the late tartaric acid biosynthesis pathway, *Vvi2-KGR* (2-keto-L-gulonic reductase,
425 *Vitvi09g00358*), was also preferentially expressed at 14 DAF before reaching a steady-state
426 level, precisely as in Jia *et al.* (2019) (Fig. 5C, 5D).

427 Three phosphoenolpyruvate carboxylases isogenes (*VviPEPC*), committed to the synthesis
428 of the malate precursor oxaloacetate, are expressed in berries, with *Vitvi12g00185*, the most
429 expressed one, belonging to the black module, correlated with the rate of malic
430 accumulation. This gene was induced at 23 DAF and peaked at the end of the lag phase at
431 47 DAF before decreasing during ripening when the net flux of malate turns negative. By
432 contrast, cytosolic malate dehydrogenases (*cytMDH*, *Vitvi07g00599*, *Vitvi07g00600*) showed
433 a constitutive expression. The mitochondrial dicarboxylate/tricarboxylate carrier
434 (*Vitvi08g01801*) was also found in the black module, suggesting a possible involvement of
435 mitochondrial MDH in the reduction of oxaloacetate to malate in the green stage. However,
436 an NADP-dependent malic enzyme (*VviNADP-ME*, *Vitvi11g00272*, yellow module) (Franke
437 and Adams, 1995; Or *et al.*, 2000) deputed in the degradation pathway showed a pyramidal

438 pattern, peaking with malic acid at the onset of ripening (51 DAF), when acidity switched
439 from accumulation to degradation. The remanence of this transcript over the complete berry
440 cycle is in line with the vital necessity of coping with cytosolic acidosis by immediately
441 degrading harmful malic acid during any transient stress, causing an H⁺ leakage from the
442 vacuole.

443

444 **Ripening guided by membrane compartmentation**

445 Although sugar import as an energy and carbon source would principally occur through the
446 symplastic pathway during the green phase (Zhang *et al.*, 2006), different sugar transporters
447 were specifically expressed, such as the bidirectional sugar transporter *VviSWEET17a*
448 (*Vitvi05g00013*), the sucrose transporter *VviSUC27* (*Vitvi18g01315*), and the hexose
449 transporter *VviHT3* (*Vitvi11g00611*). These isoforms, included in brown and black modules,
450 were then turned off at softening. Among the sucrose metabolism genes, a sucrose
451 synthase (*Vitvi11g00030*, *VviSuSy4*, green module) and a vacuolar invertase
452 (*Vitvi16g00713*, *VviGIN1* black module) were specifically expressed in the green phase
453 before decreasing during the lag phase. These genes were then suddenly replaced by the
454 plasma membrane *VviSWEET10* (*Vitvi17g00070*), the tonoplastic *VviHT6* (*Vitvi18g00056*),
455 and *VviTMT2* (*Vitvi03g00247*), marking the induction of the apoplasmic pathway (Fig. 5E,
456 5F). All these genes from the yellow module turned up at (or slightly before) softening and
457 stopped at the phloem arrest (Savoi *et al.*, 2021). *VviSWEET15* (*Vitvi01g01719*, magenta
458 module) was induced later and persisted during late ripening. A second sucrose synthase
459 (*Vitvi07g00353*, *VviSuSy3*, magenta module) started to increase at softening together with
460 three sucrose phosphate synthases (*VviSPS*, *Vitvi11g00542*, *Vitvi05g01193*, *Vitvi18g02365*)
461 from the magenta and yellow modules. *VviGIN2* (*Vitvi02g00512*) was less expressed than
462 *GIN1* in the early phase of green development and even decreased in the lag phase,
463 showing a second peak of expression during ripening.

464

465 **Proton pumps driving vacuolar hyperacidification and sugar loading**

466 The three grapevine isoforms of V-PPase showed different expression patterns (Fig. 6A,
467 6B), with *VviVPP1* (*Vitvi14g00101*) showing a rather constitutive expression profile, highly
468 expressed since the beginning of organic acid accumulation, then exhibiting a transient
469 increase simultaneous with the peak in *VviHT6* expression and malate breakdown, before
470 decreasing at 63 DAF while staying highly expressed until over-ripening. After having been
471 expressed threefold less than *VviVPP1* during the green stage, the H⁺PPase isoform *VviPP2*
472 (*Vitvi11g00560*, yellow module) suddenly reached a comparable level of about 130 RPKM
473 from the onset to the end of the sugar accumulation process, collapsing after that. Lastly, the
474 third isoform, *VviVPP3* (*Vitvi09g00693*), was quite constant during the first growth phase,

475 being at approximately 50 RPKM, for suddenly peaking at 47 DAF, a few days before
476 softening, anticipating *VviIPP2*. During the ripening phase, *VviIPP3* increased again at 58
477 DAF. The catalytic subunit A in the cytoplasmic domain V1 of V-ATPase *VviVHA-A*
478 (*Vitvi09g01397*, yellow module) and the P-ATPase *VviPH5* (*Vitvi09g00006*, grey module),
479 already expressed in the green stage, also increased in expression during the period of
480 sugar accumulation starting from softening, until declining when phloem unloading and berry
481 expansion stopped. *VviIPP2* was more expressed than *VviVHA-A*, followed by *VviPH5*, and
482 remarkably, the RPKM sum of H⁺ pumps on the tonoplast approached the huge expression
483 of *VviHT6*. In comparison, the expression of *VviPH1* (*Vitvi07g02600*), whose interaction with
484 *VviPH5* is critical for acidification in lemon, appeared negligible.

485

486 **Vacuolar ion transport**

487 Globally, a set of tonoplastic ion channels and transporters (mostly anions) reached maximal
488 expression at 51 DAF (softening, onset of ripening). The first ones, encoding for tonoplast
489 dicarboxylate transporters (*VviTDT*, *Vitvi10g00114*, *Vitvi10g00204*, yellow module), ATP-
490 binding cassette *VviABCC10* (*Vitvi15g00424*, yellow module) and *VviABCB17*
491 (*Vitvi08g01627*, tan module) were induced one week before softening (DAF 47). The
492 expression pattern of *VviTDTs* was quite parallel to the rate of malate breakdown. They were
493 followed by *VviALMT2* (*Vitvi06g00922*), then *VviALMT13* (*Vitvi02g00066*), and only during
494 late ripening by *VviALMT9* (*Vitvi17g00333*, blue module). The only transporter (*VviALMT5-6*
495 - *Vitvi01g00266*), which seemed to be faintly expressed in the green period (belonging to the
496 green module), was low expressed, questioning its central role in the entry of organic acid
497 during the accumulation period. The only tonoplastic membrane protein (*Vitvi09g00350*,
498 brown module) whose expression precisely aligns with the malate accumulation kinetics in
499 immature green berries is still uncharacterized in plants, being highly expressed in the green
500 phase for then declining at the onset of ripening and staying off during the ripening phase
501 (Fig. 6C, 6D).

502

503 **Ripening hormone signaling and transcription factors**

504 Modules pink and yellow provide precise insights into the molecular cascade triggering the
505 onset of ripening since the first one displayed the highest association with the lag phase
506 (0.73) and, in particular, the stage L5 (0.63), just four days before the second one, highly
507 correlated with the softening phase (0.77). The expression of 1-aminocyclopropane-1-
508 carboxylate oxidase (*VviACO1* - *Vitvi10g02409*, pink module), committed to ethylene
509 biosynthesis, started to increase at 23 DAF and peaked during the lag phase at 47 DAF, four
510 days before the onset of ripening. It sharply decreased after that, becoming barely
511 detectable from 58 DAF onwards (Fig. 7A, 7B). The expression of the downstream signaling

512 gene, ethylene insensitive 3 (*VviEIN3* - *Vitvi13g01126*), also increased in the green stage,
513 peaking at softening (51 DAF). However, *VviEIN3* showed a second and slightly highest
514 peak of expression at 75 DAF during late ripening stages, as previously documented
515 (Cramer *et al.*, 2014). Nevertheless, the peak of ethylene-related genes preceded the ABA-
516 related ones, and the decrease of *VviACO1* was relayed by the induction of *VviNCED2* -
517 *Vitvi10g00821*. However, *VviNCED3* - *Vitvi19g0135*, silent during the green phase, was
518 largely induced at 47 days, although reaching its maximal expression at 54 DAF, three days
519 after softening. It therefore appears as the first abrupt signal announcing softening. Notably,
520 the ABA-responsive transcription factor *VvibZIP45*, also known as *VviABF2* (*Vitvi18g00784*,
521 yellow module), showed a very close pattern with *VviNCED3* (Fig. 7A, 7B). *VviNCED2*,
522 which was two times less expressed than its homolog, suddenly increased its expression at
523 softening (51 days), peaking simultaneously with *VviNCED3* at 54 days, then decreased
524 until being switched off simultaneously with phloem unloading.

525 Based on literature data, WGCNA module membership, and gene expression profiles refined
526 by cluster analysis (Savoi *et al.*, 2023), we identified a series of transcription factors showing
527 diverse expression ranges when peaking at S7, and thus possibly involved in the fruit
528 ripening program (Fig. 7C, 7D). This included NAC members such as *VviNAC60* -
529 *Vitvi08g01843* (D'Inca *et al.*, 2023), *VviNAC26* - *Vitvi01g01038* (Zhang *et al.*, 2021),
530 *VviNAC33* - *Vitvi19g01484* (D'Inca *et al.*, 2021), a basic helix-loop-helix *VvibHLH075* -
531 *Vitvi17g00046* (Fasoli *et al.*, 2018), and *VviSEP3* - *Vitvi01g01677* from the MADS-box family
532 (Mellway and Lund, 2013). However, anion transporters and hormones previously discussed
533 prone us to look for signals activating the transcriptome reprogramming at L5, four days
534 before the phenological softening stage (Fig. 7E, 7F). This could identify a homeodomain
535 leucine zipper *VviHB13* - *Vitvi01g00958* (Terrier *et al.*, 2005), a lateral organ boundaries
536 domain gene of class I c3 *VviLDB21* - *Vitvi13g00085* (Grimplet *et al.*, 2017), a GRAS
537 transcription factor *PAT3* - *Vitvi10g00271* (Grimplet *et al.*, 2016), and finally the MADS-box
538 *AGAMOUS 2*, *VviAG2* - *Vitvi10g00663* (Mellway and Lund, 2013; Green *et al.*, 2024).

539

540 Discussion

541 For the first time, developmental changes in the net fluxes of water, hexose, malic and
542 tartaric acids were estimated and correlated to gene expression at their pertinent single fruit
543 regulatory level. Image analysis allowed us to quantify the double sigmoidal growth curves
544 non-destructively, before sampling berries with very high time resolution, considering their
545 own individualized internal clock rather than the observer one. Getting rid of the
546 developmental chimerism inherent to averaging asynchronous berries (Bigard *et al.*, 2019;
547 Shahood *et al.*, 2020) allowed us to unravel metabolically and transcriptionally

548 homogeneous phases, whose durations were shortened by two weeks at least, leading to a
549 noticeable acceleration of metabolic rates.

550

551 **Phenological transitions are faster at the onset of ripening**

552 Transitions between physiological or transcriptomic stages appeared extremely abrupt on
553 single berries, allowing us to subdivide berry development into more accurate phases than
554 previously considered. For instance, significant transcriptomic changes occurred between S6
555 and S7, which were collected three to four days apart after their individual softening dates.
556 When the incipit of softening is unknown, they remain indistinguishable in the ‘soft green’
557 class, so their transcriptomes would be averaged in phenotypically sorted or stratified
558 samples (Hernández-Montes *et al.*, 2021). The outstanding transcriptomic homogeneity of
559 single, duly sorted berries inside triplicates indicates that the comparatively large noise
560 observed on average samples (i.e., Tornielli *et al.*, 2023) reflects random variations in their
561 age pyramid. Consequently, single-berry transcriptomes led the two first PCA components to
562 explain a higher degree of variance in gene expression (54% and 15%, respectively, Fig. 2B)
563 without eliminating any noisy gene. Although some divergence appeared among triplicates
564 after the R9 stage, individual transcriptomes did not depart from the 2D plot, excluding any
565 microenvironmental scattering effect and simply indicating that the transcriptomic clock is
566 more accurate than our preliminary synchronization procedure. Actually, R9 berries were
567 sorted by considering a 50% volume increase since softening. Present results suggest that,
568 although limited in the 20 berries whose complete developmental cycle was monitored here,
569 some variation exists in berry expansion at the same developmental stage, as recently
570 indicated by high throughput automated monitoring of berry growth (Daviet *et al.*, 2023).
571 After maximum berry volume, when the berry turns hydraulically disconnected from the vine
572 (Savoi *et al.*, 2021) (Fig. 4A, 4B), the transcriptome turns quite stable: R10 and Sh11
573 samples are grouped (Fig. 2B), as the transcriptomic time decelerated (Fig. 2C).

574 The spatiotemporal separation in the synthesis of specific phenolic compounds is well
575 documented during grape berry development (Czemmel *et al.*, 2012). Proanthocyanidin
576 biosynthesis is modulated by *VviMYBPA1* and *VviMYBPA2* (Bogs *et al.*, 2007; Terrier *et al.*,
577 2009). Single berry transcriptomics allows to resolve the expression patterns of these TF just
578 separated a few days apart. In this respect, *VviMYBPA2* strongly decreased before 23 DAF,
579 simultaneously with the core PA gene *VviLAR1* and *VviANR*. Moreover, among other water
580 channels isogenes, *VviPIP2.7* and *VviTIP2.1* were predominantly expressed during the first
581 growth phase, when water would be principally translocated by xylem and the limited phloem
582 unloading essentially symplastic, thereby excluding any significant transmembrane fluxes in
583 sieve tubes (Fig. 4B). *VviPIP2.7* was replaced by *VviPIP2.5* and *VviPIP2.3*, when shifting to
584 predominating apoplastic phloem unloading, being these isoforms most probably sieve

585 element-specific (Stanfield *et al.*, 2017). Conversely, *VviPIP1.3* showed a comparable and
586 constitutive expression from 23 DAF until the phloem stopped, possibly ensuring continuous
587 water movement inside the flesh. *VviTIP1.2*, reaching during ripening the same RPKM as
588 *VviTIP2.1* during the green stage, may thus also appear ‘flesh specific’. The adaptive
589 mechanisms below this shift in TIP isogenes is questionable. Finally, RPKM of expansin
590 genes indicated a higher expression during ripening of *VviEXP14* and *VviEXP19* compared
591 to other isogene expressed in green phase, Fig. 4D, (Malacarne *et al.*, 2024). The two
592 successive waves of expansin activated during ripening may respectively correspond to
593 softening and growth resumption.

594

595 **Sequence of ripening events**

596 Kinetic analysis of individual berries confirms that the first ripening phenotype indicators are
597 a drop in fruit firmness and a reduction in the G/F ratio (Fig. 1C). These changes co-
598 occurred with a pronounced surge in the intake and storage of photoassimilates above 0.15
599 M hexoses, as previously documented (Bigard *et al.*, 2022). *VviSWEET10* and *VviHT6* sugar
600 transporters induced on the plasma and tonoplast membranes between 47 and 51 DAF
601 announced this huge acceleration of phloem unloading. Berries analyzed through continuous
602 image analysis softened at 51 ± 1.5 DAF, with a notable 7 ± 1.5 days delay before growth
603 resumption. This early ripening sequence agrees with previous reports on which growth was
604 lacking (Gouthu and Deluc, 2015; Castellarin *et al.*, 2016; Hernández-Montes *et al.*, 2021)
605 and confirms that berry expansion would not resume before 0.4 M hexoses (Shahood *et al.*,
606 2020). Clearly, single berry monitoring allows distinguishing developmental events
607 previously thought to be simultaneous in all studies based on average samples and
608 considering 50% of colored berries (mid-veraison) as a rough indicator of the onset of
609 ripening (i.e., Fasoli *et al.*, 2018; Theine *et al.*, 2021). In fact, both *VviMYBA1*, *VviMYBA2*
610 TFs and the regulatory gene *VviUFGT* simultaneously enhanced their expression at 54 DAF,
611 four days after softening (Fig. 5A, 5B). Recently, the first high throughput automated analysis
612 of time-lapse images led to the conclusion that there is a 4.4-day average delay between
613 growth resumption and coloration in the *Vitis vinifera* cv Alexandrouli (Daviet *et al.*, 2023).
614 However, one should not reject that this delay should depend on genetic differences and
615 environmental conditions like light, water, or crop load. Merging growth and sugar
616 concentration data evidenced a progressive acceleration in hexose accumulation at
617 softening, finally reaching $64 \mu\text{mol hexose} \cdot \text{min}^{-1} \text{ N berry}^{-1}$. This net accumulation rate is
618 four times faster than can be calculated on average, unsynchronized berry samples (i.e., 16
619 $\mu\text{mol hexose} \cdot \text{min}^{-1} \text{ N berry}^{-1}$ for a 6-week period in Fasoli *et al.*, (2018)). Malic acid
620 breakdown started at approximately 0.3 M sugar (Fig. 1D), and the initial rate of malate

621 breakdown corresponds to a 1:1 exchange of sucrose for H⁺ at the tonoplast (Fig. S1), as
622 recently shown on different varieties (Shahood *et al.*, 2020; Bigard *et al.*, 2022).

623 Fruit ripening involves various hormonal signals and interactions with several TFs. As a non-
624 climacteric fruit, ripening relies on a composite interplay among hormones at the onset of
625 ripening, among which the ABA/auxin balance and ethylene (Zenoni *et al.*, 2023). Increased
626 time resolution gained on single berry put forwards *VviNCED3*, the main ABA biosynthetic
627 gene, as a first abrupt signal preceding the drop in the ethylene *VviACO1* and the induction
628 of *VviNCED2* at softening (Fig. 7B). Finally, several transcription factors already
629 characterized in the literature appeared clearly induced shortly before softening (Fig. 7E, 7F)
630 or peaking a few days later at S7 (Fig. 7C, 7D). Further studies are needed to establish their
631 respective roles in fruit ripening.

632

633 **Unraveling the central role of vacuolar energetics on the sugar acid nexus**

634 The net sugar accumulation and malate breakdown rates are consistent with the sudden
635 induction of global sucrose/H⁺ exchange at the onset of ripening when the
636 *VviHT6/VviSWEET10* transcripts are switched on. The rate of sugar accumulation
637 progressively accelerates like the expression of *VviSWEET15*; however, unlike the previous
638 ones, this last gene is not switched off at the completion of sugar loading. Sugar storage
639 would be first energized by the vacuolar acidity gradient previously formed in the green
640 stage upon malic acid accumulation, the sugar/H⁺ exchange being charge compensated by
641 the release of vacuolar malate, triggering its mitochondrial oxidation. However, sugar loading
642 accelerates while the malic acid content vanishes (Fig. S1). Transport experiments on
643 tonoplast vesicles suggested that H⁺ pumps progressively take over the electro-
644 neutralization process (Terrier *et al.*, 2001). The simultaneous increase of *VviHT6* and
645 vacuolar H⁺ pump transcripts (Fig. 6A, 6B) suggests that a phenotypically silent H⁺
646 recirculation could parallel or relay malate efflux. This could indicate that (1) hexose, not
647 sucrose, would be transported by *VviHT6*, (2) a fail-safe mechanism preventing excessive
648 release of acidity when compared to oxidative capacity must be anticipated to provide instant
649 response upon stress, or (3) due to a lack of selectivity of the malate channel, non-
650 metabolizable tartrate is released in the cytoplasm, requiring an active return to the vacuole.
651 Whatever, with a respiratory rate of about 12-20 μmolO₂min⁻¹Nberry⁻¹, hence a maximum of
652 110 μmol ATP min⁻¹Nberry⁻¹ (recalculated from Morales *et al.*, 2024), the intensity of sugar
653 loading in individual berries turns hardly compatible with the energetics of a classical import
654 pathway involving cell wall sucrose inversion and hexose H⁺ symporter at the plasma
655 membrane, which would consume circa 55% of ATP possibly formed by oxidative
656 phosphorylation. This energy-guzzling pathway is thus bypassed by *VviSWEET10*
657 expression, which closely matches the one of *VviHT6*, preceding the one of *VviSWEET15*.

658

659 **Tonoplasmic proton pumps are simultaneously expressed**

660 Within fruit cells, malate primarily undergoes synthesis in the cytoplasm, a process achieved
661 by PEPC, MDH, and possibly malate synthase (Sweetman *et al.*, 2009; Etienne *et al.*, 2013).
662 Its synthesis from hexose also produces two harmful H⁺ retro-controlling PEPcase and
663 activating ME, so H⁺ transport from the cytoplasm into vacuoles pulls the overall malate
664 synthesis and accumulation process. Here, we confirm that the three types of H⁺ pumps are
665 simultaneously expressed on the tonoplast (V-PPases, V-ATPases, and P-ATPases) in
666 green and ripe berries. The co-expression of these pumps in the green stage apparently
667 contradicts the thermodynamic need to replace V-PPases and V-ATPase with a more
668 electrogenic P-ATPase complex evoked on citrus (Strazzer *et al.*, 2019). As a matter of fact,
669 the *in vivo* ΔG_{PPi} of -27.3 kJ/mol (Davies *et al.*, 1993) allows a 1H⁺/PPi PPIase to form a 4.7
670 pH units gradient across the tonoplast, and a similar conclusion holds for a 2H⁺/ATP
671 vATPase fed with ΔG_{ATP} of -54.6 kJ/mol. These conservative values (nmin:1.75 H⁺/ATP; max
672 oxphos ΔG_{MgATP} : -61+/-3 kJ/mol) (Roberts *et al.*, 1985; Davies *et al.*, 1994; Wiseman *et al.*,
673 2023) indicate there is no strict requirement for more electrogenic H⁺ pump in green berries
674 at pH_v=2.7. PH5, erroneously considered a contaminant in Terrier *et al.* (1998), is also co-
675 expressed with V-ATPase in apples and pears and promotes malate accumulation in these
676 fruits (Song *et al.*, 2022; Huang *et al.*, 2023). The presence of two ATPases with possibly
677 different H/ATP ratios is puzzling, so further work is needed to establish whether these
678 pumps operate in parallel on the same membrane.

679

680 **Open questions on malate entry and exit route**

681 Questions remain on malate's entry and exit route to and from the vacuole. Unexpectedly,
682 the expression of *bona fide* malate/tartrate vacuolar transporters did not parallel the vacuolar
683 influx of organic acids during the green stage (Fig. 6C 6D), neither that of many other anion
684 transporters. The only aluminum-activated malate transporter family member characterized
685 in grapevine is *VviALMT9* on chromosome 17. This gene has been pointed out to mediate
686 inward-rectifying malate and tartrate currents across the tonoplast (De Angeli *et al.*, 2013).
687 However, its expression during ripening does not match the timing of malate entry in the
688 green phase (Fig. 6C 6D). ALMT phylogenetic analysis (Dreyer *et al.*, 2012; Rienh *et al.*,
689 2016) indicated that additional clade II genes should be targeted to berry tonoplast, such as
690 the *VviALMT5-6*, misannotated as a unique gene in the grapevine chromosome 1. Similarly,
691 on apple chromosome 16, the Ma locus comprises two genes, *MDP244294* and
692 *MDP252114* (Bai *et al.*, 2012), sharing the same conserved duplicated structure as in *V.*
693 *vinifera* chromosome 1, and not on chromosome 17. Therefore, alignment and synteny
694 confirm *VviALMT5-6* as the true orthologs of the Ma locus, controlling the genetic diversity of

695 malic acid in apple. Actually, these ALMT were constitutively but slightly expressed during
696 pericarp development, prompting the question of the effective player of malate vacuolar
697 import during the green stage. In this respect, by digging the present dataset, we identified a
698 new putative anion channel still uncharacterized in plants (*Vitvi09g00350*, *D7U0H3*) whose
699 expression perfectly matched the malate accumulation kinetics in green berries. Although
700 unmentioned, such expression profile is confirmed in other transcriptomic data (i.e., Rienth
701 *et al.*, 2016; Fasoli *et al.*, 2018). Finally, the transporter was localized on the vacuole during
702 a pioneering proteomic study and highly abundant during the green phase (Kuang *et al.*,
703 2019). Further studies are needed to characterize the functional role of this gene.

704

705 **Conclusion**

706 Our work provides a resource on single-fruit transcriptomes and paves the way for
707 comparative analyses of acidic fleshy fruit development in different perennial species, both
708 non-climacteric and beyond. Time-resolved changes in gene expression highlighted the
709 multi-faceted adaptation of membrane transports retained by evolution, allowing the pericarp
710 to rapidly import sugar, before signaling this nutritional reward with anthocyanin. Announced
711 by concerted changes in hormonal, kinetic, and membrane transporters a few days before
712 softening, the sudden induction of sugar accumulation and malate breakdown coincides with
713 the activation of vacuolar transport systems (*VviHT6* and *VviSWEET10*) at the onset of
714 ripening. The findings suggest a model where the vacuolar acidity gradient drives sugar
715 storage while triggering the malate oxidation processes and the actuation of H⁺ pumps.
716 Finally, known ALMT transporters, like *VviALMT9*, do not align with malate accumulation in
717 the green phase. Instead, an uncharacterized anion transporter (*Vitvi09g00350*) was
718 strongly expressed, hinting at its potential role in vacuolar malate import. Further functional
719 studies are needed to clarify the role of specific transporters in malate movement during
720 berry development.

721

722 **Supplementary materials**

723 Figure S1. The stoichiometry of malic acid breakdown versus sugar accumulation.

724 Table S1. List of samples, their acronym, and phenological stages of reference.

725 Table S2. Parameters of the sigmoidal function calculation for the 20 reference berries.

726 Table S3. Reconstructed curve of berry growth

727 Table S4. Metabolite fluxes in every berries sampled from anthesis to overripening.

728 Table S5. Top-100 PC1 and PC2 positive and negative loadings.

729 Table S6. Transcriptomics time.

730 Table S7. WGCNA gene module association and membership correlation.

731

732 **Acknowledgments**

733 We thank the Department of Viticulture and Enology of Institut Agro Montpellier for
734 facilitating access to the experimental vineyard Pierre Galet and the genomic platform of
735 INRAE managed by Dr. Sylvain Santoni for helping in sample preparation and processing.

736

737 **Author contributions**

738 LT and CR: conceptualization; SS, LT, and CR: methodology; SS, MS, GS, AW: formal
739 analysis; SS, LT, and CR: investigation; SS, LT, and CR: resources; SS and CR: data
740 curation; SS: writing - original draft; SS, LT, and CR: writing - review & editing; LT: funding
741 acquisition.

742

743 **Conflict of interest**

744 The authors declare no conflict of interest.

745

746 **Funding**

747 This work was financially supported by the Foundation Jean Poupelain (Javresac, France),
748 the Agence Nationale de la Recherche (ANR, G2WAS project, ANR-19-CE20-0024), the
749 Comité Interprofessionnel des Vins de Bordeaux (CIVB), the Institut Agro Montpellier, the
750 University of Torino with the Grant for Internationalization 2022.

751

752 **Data Availability**

753 All raw transcriptomic reads have been deposited in the NCBI Sequence Read Archive
754 (<http://www.ncbi.nlm.nih.gov/sra>). The BioProject is PRJNA862686.

References

Amato A, Cavallini E, Walker AR, et al. 2019. The MYB5-driven MBW complex recruits a WRKY factor to enhance the expression of targets involved in vacuolar hyper-acidification and trafficking in grapevine. *The Plant Journal* **99**, 1220–1241.

Anders S, Pyl PT, Huber W. 2015. HTSeq—a Python framework to work with high-throughput sequencing data. *Bioinformatics* **31**, 166–169.

Bai Y, Dougherty L, Li M, Fazio G, Cheng L, Xu K. 2012. A natural mutation-led truncation in one of the two aluminum-activated malate transporter-like genes at the Ma locus is associated with low fruit acidity in apple. *Molecular Genetics and Genomics* **287**, 663–678.

Batista-Silva W, Nascimento VL, Medeiros DB, Nunes-Nesi A, Ribeiro DM, Zsögön A, Araújo WL. 2018. Modifications in organic acid profiles during fruit development and ripening: correlation or causation? *Frontiers in Plant Science* **9**.

Becker FN, Fink AH, Bissolli P, Pinto JG. 2022. Towards a more comprehensive

assessment of the intensity of historical European heat waves (1979–2019). *Atmospheric Science Letters*, e1120.

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

Bigard A, Romieu C, Sire Y, Torregrosa L. 2020. *Vitis vinifera* L. diversity for cations and acidity is suitable for breeding fruits coping with climate warming. *Frontiers in Plant Science* **11**.

Bigard A, Romieu C, Sire Y, Veyret M, Ojeda H, Torregrosa L. 2019. The kinetics of grape ripening revisited through berry density sorting. *OENO One* **53**.

Bogs J, Jaffé FW, Takos AM, Walker AR, Robinson SP. 2007. The grapevine transcription factor VvMYBPA1 regulates proanthocyanidin synthesis during fruit development. *Plant Physiology* **143**, 1347–1361.

Bolger AM, Lohse M, Usadel B. 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* **30**, 2114–2120.

Butelli E, Licciardello C, Ramadugu C, Durand-Hulak M, Celant A, Reforgiato Recupero G, Froelicher Y, Martin C. 2019. Noemi Controls Production of Flavonoid Pigments and Fruit Acidity and Illustrates the Domestication Routes of Modern Citrus Varieties. *Current Biology* **29**, 158-164.e2.

Canaguier A, Grimplet J, Di Gaspero G, et al. 2017. A new version of the grapevine reference genome assembly (12X.v2) and of its annotation (VCost.v3). *Genomics Data* **14**, 56–62.

Castellarin SD, Gambetta GA, Wada H, Krasnow MN, Cramer GR, Peterlunger E, Shackel KA, Matthews MA. 2016. Characterization of major ripening events during softening in grape: turgor, sugar accumulation, abscisic acid metabolism, colour development, and their relationship with growth. *Journal of Experimental Botany* **67**, 709–722.

Cramer GR, Ghan R, Schlauch KA, Tillett RL, Heymann H, Ferrarini A, Delledonne M, Zenoni S, Fasoli M, Pezzotti M. 2014. Transcriptomic analysis of the late stages of grapevine (*Vitis vinifera* cv. Cabernet Sauvignon) berry ripening reveals significant induction of ethylene signaling and flavor pathways in the skin. *BMC Plant Biology* **14**, 1–21.

Czemmel S, Heppel SC, Bogs J. 2012. R2R3 MYB transcription factors: key regulators of the flavonoid biosynthetic pathway in grapevine. *Protoplasma* **249**, 109–118.

Davies C, Burbidge CA, Böttcher C, Dodd AN. 2023. Loss of Diel Circadian Clock Gene Cycling Is a Part of Grape Berry Ripening. *Plant and Cell Physiology*, pcd099.

Davies JM, Hunt I, Sanders D. 1994. Vacuolar H(+)-pumping ATPase variable transport coupling ratio controlled by pH. *Proceedings of the National Academy of Sciences of the United States of America* **91**, 8547–8551.

Davies JM, Poole RJ, Sanders D. 1993. The computed free energy change of hydrolysis of inorganic pyrophosphate and ATP: apparent significance for inorganic-pyrophosphate-driven reactions of intermediary metabolism. *Biochimica et Biophysica Acta (BBA) - Bioenergetics* **1141**, 29–36.

Daviet B, Fournier C, Cabrera-Bosquet L, Simonneau T, Cafier M, Romieu C. 2023. Ripening dynamics revisited: an automated method to track the development of asynchronous berries on time-lapse images. *Plant Methods* **19**, 146.

De Angeli A, Baetz U, Francisco R, Zhang J, Chaves MM, Regalado A. 2013. The vacuolar channel VvALMT9 mediates malate and tartrate accumulation in berries of *Vitis vinifera*. *Planta* **238**, 283–291.

- DeBolt S, Cook DR, Ford CM.** 2006. L-Tartaric acid synthesis from vitamin C in higher plants. *Proceedings of the National Academy of Sciences* **103**, 5608–5613.
- D’Incà E, Cazzaniga S, Foresti C, Vitulo N, Bertini E, Galli M, Gallavotti A, Pezzotti M, Battista Torielli G, Zenoni S.** 2021. VviNAC33 promotes organ de-greening and represses vegetative growth during the vegetative-to-mature phase transition in grapevine. *New Phytologist* **231**, 726–746.
- D’Incà E, Foresti C, Orduña L, et al.** 2023. The transcription factor VviNAC60 regulates senescence- and ripening-related processes in grapevine. *Plant Physiology*, kiad050.
- Dreyer I, Gomez-Porras JL, Riaño Pachón DM, Hedrich R, Geiger D.** 2012. Molecular Evolution of Slow and Quick Anion Channels (SLACs and QUACs/ALMTs). *Frontiers in Plant Science* **3**.
- Etienne A, Génard M, Lobit P, Mbéguié-A-Mbéguié D, Bugaud C.** 2013. What controls fleshy fruit acidity? A review of malate and citrate accumulation in fruit cells. *Journal of Experimental Botany* **64**, 1451–1469.
- Fasoli M, Richter CL, Zenoni S, Bertini E, Vitulo N, Santo SD, Dokoozlian N, Pezzotti M, Torielli GB.** 2018. Timing and order of the molecular events marking the onset of berry ripening in grapevine. *Plant Physiology* **178**, 1187–1206.
- Fasoli M, Santo SD, Zenoni S, et al.** 2012. The grapevine expression atlas reveals a deep transcriptome shift driving the entire plant into a maturation program. *The Plant Cell* **24**, 3489–3505.
- Franke KE, Adams DO.** 1995. Cloning of a full-length cDNA for malic enzyme (EC 1.1.1.40) from grape berries. *Plant Physiology* **107**, 1009–1010.
- Frenzke L, Röckel F, Wenke T, et al.** 2024. Genotyping-by-sequencing-based high-resolution mapping reveals a single candidate gene for the grapevine veraison locus Ver1. *Plant Physiology*, kiae272.
- Gouthu S, Deluc LG.** 2015. Timing of ripening initiation in grape berries and its relationship to seed content and pericarp auxin levels. *BMC Plant Biology* **15**, 46.
- Green E, Shmuleviz R, Amato A, Torielli GB, Dokoozlian N, Fasoli M.** 2024. Unraveling the key molecular events of Pinot noir berry ripening under varying crop load. *Scientia Horticulturae* **338**, 113644.
- Grimplet J, Agudelo-Romero P, Teixeira RT, Martinez-Zapater JM, Fortes AM.** 2016. Structural and functional analysis of the GRAS gene family in grapevine indicates a role of GRAS proteins in the control of development and stress responses. *Frontiers in Plant Science* **7**.
- Grimplet J, Pimentel D, Agudelo-Romero P, Martinez-Zapater JM, Fortes AM.** 2017. The LATERAL ORGAN BOUNDARIES Domain gene family in grapevine: genome-wide characterization and expression analyses during developmental processes and stress responses. *Scientific Reports* **7**, 15968.
- Hernández-Montes E, Zhang Y, Chang B-M, Shcherbatyuk N, Keller M.** 2021. Soft, sweet, and colorful: stratified sampling reveals sequence of events at the onset of grape ripening. *American Journal of Enology and Viticulture* **72**, 137–151.
- Hichri I, Heppel SC, Pillet J, Léon C, Czemplin S, Delrot S, Lauvergeat V, Bogs J.** 2010. The basic Helix-Loop-Helix transcription factor MYC1 is involved in the regulation of the flavonoid biosynthesis pathway in grapevine. *Molecular Plant* **3**, 509–523.
- Huang X-Y, Xiang Y, Zhao Y-W, Wang C-K, Wang J-H, Wang W-Y, Liu X-L, Sun Q, Hu D-G.** 2023. Regulation of a vacuolar proton-pumping P-ATPase MdPH5 by MdMYB73 and its role in malate accumulation and vacuolar acidification. *aBIOTECH* doi: 10.1007/s42994-023-00115-7.

- Jia Y, Burbidge CA, Sweetman C, Schutz E, Soole K, Jenkins C, Hancock RD, Bruning JB, Ford CM.** 2019. An aldo-keto reductase with 2-keto-l-gulonate reductase activity functions in l-tartaric acid biosynthesis from vitamin C in *Vitis vinifera*. *Journal of Biological Chemistry* **294**, 15932–15946.
- Kim D, Langmead B, Salzberg SL.** 2015. HISAT: a fast spliced aligner with low memory requirements. *Nature Methods* **12**, 357–360.
- Kuang L, Chen S, Guo Y, Ma H.** 2019. Quantitative proteome analysis reveals changes in the protein landscape during grape berry development with a focus on vacuolar transport proteins. *Frontiers in Plant Science* **10**.
- Langfelder P, Horvath S.** 2008. WGCNA: an R package for weighted correlation network analysis. *BMC Bioinformatics* **9**, 559.
- Malacarne G, Lagreze J, Rojas San Martin B, Malnoy M, Moretto M, Moser C, Dalla Costa L.** 2024. Insights into the cell-wall dynamics in grapevine berries during ripening and in response to biotic and abiotic stresses. *Plant Molecular Biology* **114**, 38.
- Martinoia E, Maeshima M, Neuhaus HE.** 2007. Vacuolar transporters and their essential role in plant metabolism. *Journal of Experimental Botany* **58**, 83–102.
- Melino VJ, Soole KL, Ford CM.** 2009. Ascorbate metabolism and the developmental demand for tartaric and oxalic acids in ripening grape berries. *BMC Plant Biology* **9**, 145.
- Mellway RD, Lund ST.** 2013. Interaction analysis of grapevine MIKCC-type MADS transcription factors and heterologous expression of putative *véraison* regulators in tomato. *Journal of Plant Physiology* **170**, 1424–1433.
- Morales F, Cabodevilla A, Pascual I, Urdiain A.** 2024. New instrumentation in grapevine research: A dual respiration prototype for grape berries and whole bunch. The grape CO₂/O₂ respiratory quotient revisited. *Computers and Electronics in Agriculture* **217**, 108659.
- Neves C, Ribeiro B, Amaro R, Expósito J, Grimplet J, Fortes AM.** 2023. Network of GRAS transcription factors in plant development, fruit ripening and stress responses. *Horticulture Research* **10**, uhad220.
- Niimi Y, Torikata H.** 1979. Changes in Photosynthesis and Respiration during Berry Development in Relation to the Ripening of Delaware Grapes. *Journal of the Japanese Society for Horticultural Science* **47**, 448–453.
- Nueda MJ, Tarazona S, Conesa A.** 2014. Next maSigPro: updating maSigPro bioconductor package for RNA-seq time series. *Bioinformatics* **30**, 2598–2602.
- Ojeda H, Deloire A, Carbonneau A, Ageorges A, Romieu C.** 1999. Berry development of grapevines: Relations between the growth of berries and their DNA content indicate cell multiplication and enlargement. *VITIS - Journal of Grapevine Research* **38**, 145–145.
- Or E, Baybik J, Sadka A, Saks Y.** 2000. Isolation of mitochondrial malate dehydrogenase and phosphoenolpyruvate carboxylase cDNA clones from grape berries and analysis of their expression pattern throughout berry development. *Journal of Plant Physiology* **157**, 527–534.
- Palumbo MC, Zenoni S, Fasoli M, Massonnet M, Farina L, Castiglione F, Pezzotti M, Paci P.** 2014. Integrated network analysis identifies fight-club nodes as a class of hubs encompassing key putative switch genes that induce major transcriptome reprogramming during grapevine development. *The Plant Cell* **26**, 4617–4635.
- Pérez-Díaz R, Ryngajllo M, Pérez-Díaz J, Peña-Cortés H, Casaretto JA, González-Villanueva E, Ruiz-Lara S.** 2014. VvMATE1 and VvMATE2 encode putative proanthocyanidin transporters expressed during berry development in *Vitis vinifera* L. *Plant Cell Reports* **33**, 1147–1159.

- Perotti MF, Posé D, Martín-Pizarro C.** 2023. Non-climacteric fruit development and ripening regulation: ‘the phytohormones show’. *Journal of Experimental Botany*, erad271.
- Rauf A, Imran M, Abu-Izneid T, Ihtisham-UI-Haq, Patel S, Pan X, Naz S, Sanches Silva A, Saeed F, Rasul Suleria HA.** 2019. Proanthocyanidins: A comprehensive review. *Biomedicine & Pharmacotherapy* **116**, 108999.
- Reluy NP, Baghdadi N, Simonneau T, Bazzi H, Hajj ME, Pret V, Amin G, Daret E.** 2022. Can we detect the damage of a heatwave on vineyards using Sentinel-2 optical remote sensing data? *OENO One* **56**, 145–159.
- Rienth M, Torregrosa L, Luchaire N, Chatbanyong R, Lecourieux D, Kelly MT, Romieu C.** 2014. Day and night heat stress trigger different transcriptomic responses in green and ripening grapevine (*Vitis vinifera*) fruit. *BMC Plant Biology* **14**, 108.
- Rienth M, Torregrosa L, Sarah G, Ardisson M, Brillouet J-M, Romieu C.** 2016. Temperature desynchronizes sugar and organic acid metabolism in ripening grapevine fruits and remodels their transcriptome. *BMC Plant Biology* **16**, 164.
- Roberts JKM, Lane AN, Clark RA, Nieman RH.** 1985. Relationships between the rate of synthesis of ATP and the concentrations of reactants and products of ATP hydrolysis in maize root tips, determined by ³¹P nuclear magnetic resonance. *Archives of Biochemistry and Biophysics* **240**, 712–722.
- 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. *Horticulture Research* **8**, 1–15.
- Savoi S, Torregrosa L, Romieu C.** 2023. Exploring the mechanisms of grapevine single berry development and ripening. *IVES Conference Series, GiESCO 2023*.
- Shahood R, Torregrosa L, Savoi S, Romieu C.** 2020. First quantitative assessment of growth, sugar accumulation and malate breakdown in a single ripening berry. *OENO One* **54**, 1077–1092.
- Song J, Chen Y, Lu Z, Zhao G, Wang X, Zhai R, Wang Z, Yang C, Xu L.** 2022. PbPH5, an H⁺ P-ATPase on the tonoplast, is related to malic acid accumulation in pear fruit. *Journal of Integrative Agriculture* **21**, 1645–1657.
- Stanfield RC, Hacke UG, Laur J.** 2017. Are phloem sieve tubes leaky conduits supported by numerous aquaporins? *American Journal of Botany* **104**, 719–732.
- Strazzer P, Spelt CE, Li S, Bliet M, Federici CT, Roose ML, Koes R, Quattrocchio FM.** 2019. Hyperacidification of Citrus fruits by a vacuolar proton-pumping P-ATPase complex. *Nature Communications* **10**, 744.
- Sweetman C, Deluc LG, Cramer GR, Ford CM, Soole KL.** 2009. Regulation of malate metabolism in grape berry and other developing fruits. *Phytochemistry* **70**, 1329–1344.
- Tan YZ, Keon KA, Abdelaziz R, Imming P, Schulze W, Schumacher K, Rubinstein JL.** 2022. Structure of V-ATPase from citrus fruit. *Structure* **30**, 1403-1410.e4.
- Terrier N, Deguilloux C, Sauvage F-X, Martinoia E, Romieu C.** 1998. Proton pumps and anion transport in *Vitis vinifera*: The inorganic pyrophosphatase plays a predominant role in the energization of the tonoplast. *Plant Physiology and Biochemistry* **36**, 367–377.
- Terrier N, Glissant D, Grimplet J, et al.** 2005. Isogene specific oligo arrays reveal multifaceted changes in gene expression during grape berry (*Vitis vinifera* L.) development. *Planta* **222**, 832–847.
- Terrier N, Sauvage F-X, Ageorges A, Romieu C.** 2001. Changes in acidity and in proton transport at the tonoplast of grape berries during development. *Planta* **213**, 20–28.
- Terrier N, Torregrosa L, Ageorges A, Vialet S, Verriès C, Cheynier V, Romieu C.** 2009.

Ectopic expression of VvMYBPA2 promotes proanthocyanidin biosynthesis in grapevine and suggests additional targets in the pathway. *Plant Physiology* **149**, 1028–1041.

Theine J, Holtgräwe D, Herzog K, Schwander F, Kicherer A, Hausmann L, Viehöver P, Töpfer R, Weisshaar B. 2021. Transcriptomic analysis of temporal shifts in berry development between two grapevine cultivars of the Pinot family reveals potential genes controlling ripening time. *BMC Plant Biology* **21**, 327.

Tornielli GB, Sandri M, Fasoli M, Amato A, Pezzotti M, Zuccolotto P, Zenoni S. 2023. A molecular phenology scale of grape berry development. *Horticulture Research* **10**, uhad048.

Wiseman RW, Brown CM, Beck TW, Brault JJ, Reinoso TR, Shi Y, Chase PB. 2023. Creatine Kinase Equilibration and Δ GATP over an Extended Range of Physiological Conditions: Implications for Cellular Energetics, Signaling, and Muscle Performance. *International Journal of Molecular Sciences* **24**, 13244.

Zenoni S, Savoi S, Busatto N, Tornielli GB, Costa F. 2023. Molecular regulation of apple and grape ripening: exploring common and distinct transcriptional aspects of representative climacteric and non-climacteric fruits. *Journal of Experimental Botany*, erad324.

Zhang S, Dong R, Wang Y, Li X, Ji M, Wang X. 2021. NAC domain gene VvNAC26 interacts with VvMADS9 and influences seed and fruit development. *Plant Physiology and Biochemistry* **164**, 63–72.

Zhang XY, Wang XL, Wang XF, Xia GH, Pan QH, Fan RC, Wu FQ, Yu XC, Zhang DP. 2006. A shift of phloem unloading from symplasmic to apoplasmic pathway is involved in developmental onset of ripening in grape berry. *Plant Physiology* **142**, 220–232.

Zhu J, Génard M, Poni S, Gambetta GA, Vivin P, Vercambre G, Trought MCT, Ollat N, Delrot S, Dai Z. 2019. Modelling grape growth in relation to whole-plant carbon and water fluxes. *Journal of Experimental Botany* **70**, 2505–2521.

Figure legends

Figure 1. Growth and major osmotica in synchronized berries. **A:** Growth kinetics of 20 single Syrah berries (black lines). The maximal volumes were normalized, and then the first growth periods were synchronized upon shifting the individual DAF. Experimental data (black lines) were fitted to a double logistic function (red line): $v=0.44506/(1+\exp((23.14-\text{time})/4.78848)) + 0.55494/(1+\exp((62.05-\text{time})/3.31967))$, Table S3. The berry developmental phases are named according to the different shades of gray. **B:** Growth kinetics and accumulation of major osmotica in synchronized berries, from flowering to over-ripening. For comparative purposes, units relate to N=485 berries, yielding 1 Kg of fruit for variety Syrah at maximum volume. Green and gray dots represent malic and tartaric acids, while yellow and blue represent glucose+fructose and potassium, respectively. The detailed fluxes dataset is available in Table S4. The red line reports the fitted double sigmoidal growth curve established in Fig. 1A. **C:** Sugar accumulation vs. the ratio of the two hexoses. Growth resumption occurs later (at 0.4M) than softening and the onset of sugar accumulation. Squares represent the evolution of the glucose/fructose ratio, while circles show berry expansion. **D:** Sugar accumulation vs. malate breakdown. L5 green hard, S6-S7

green soft, R8-R9-R10-Sh11 red to black. At Sh11, the decrease in berry volume was not accompanied by visible berry wrinkling yet.

Figure 2. Transcriptomics clock overview. **A:** Repartition of the eleven developmental stages subjected to RNA-Sequencing according to berry growth phases. **B:** PCA of gene expression in 33 single berries sampled at 11 developmental stages, as color legend specifies. G: green phase, L: lag phase (green hard), S: softening (green soft), R: ripening red-purple, and Sh: shriveling. Green phase: G1 to L5, Ripening: S6 to R10, Over Ripening: Sh11. **C:** Transcriptomics time (red line, Table S6) is inferred from the curvilinear distance of the fitted line on the PCA (inlet).

Figure 3. Weighted Gene Coexpression Analysis results. **A:** Hierarchical clustering tree dendrogram of 6374 time-modulated genes, with dissimilarity calculated with a soft thresholding power β of 30 based on topological overlap matrix. Each leaf (black vertical lines) corresponds to a gene. Branches of the dendrogram group together densely interconnected, highly co-expressed genes assigned 13 module colors, visible on the bottom bar. **B:** The Eigengene dendrogram shows the modules' distribution and interrelationships. ME = Module Eigengene. **C** and **D:** Module-Trait relationships and Module-Stage relationships, respectively. Each row corresponds to a module, and each column corresponds to a trait specified at the bottom. Numbers inside the cell describe the correlation coefficient and the associated p-value in brackets. Red and blue colors indicate a positive or negative correlation between the trait or stage and the module. Modules are listed on the right sidebar column.

Figure 4. Fruit growth and expansion. Aquaporin (**A, B**) and expansin (**C, D**) genes active in the first period of water entry and growth phase as *VviPIP2.7*, *VviPIP2.4*, *VviTIP2.1*, *VviTIP1.1*, *VviEXPA16* and *VviEXPB02* and the isogenes active in the second phase of growth as *VviPIP2.5*, *VviPIP2.3*, *VviTIP1.2*, *VviTIP1.3*, *VviEXPA14*, *VviEXPA19*, and *VviEXPA18*. In addition, two more expansion genes were identified with an increasing trend during the shriveling time: *VviEXPA01* and *VviEXPB04*. Lastly, two aquaporins were active in both green and ripening phases (*VviPIP1.3* and *VviPIP1.4*). Graphs are expressed as surface normalized data (A, C) and Reads Per Kilobase per Million mapped reads (RPKM) (B, D). In the surface normalized graphs, each expression value was divided by the average on the 11 developmental points to reject large differences in expression among genes. Softening (S) started at an unknown date between 47 and 51 days.

Figure 5. Key genes involved in proanthocyanidins and osmolytes biosynthesis and transport. *VviLAR1*, *VviANR*, and *VviMYBPA2* genes of the flavonol pathway are expressed at the very early phase of berry development, followed by *VviLAR2* and *VviMYBPA1* in the green plateau; anthocyanins-related genes as *VviMYBA1*, *VviMYBA2*, and *VviUFGT* are activated three days after berry softening (**A, B**). *VviVTC2*, *VviL-IDH*, and *Vvi2-KGR* are known biosynthetic genes of tartaric acid, while *VviPEPC*, *VviMDH*, and *VviNADP-ME* are committed to malic acid synthesis and breakdown (**C, D**). ‘Green’ sugar transporters such as *VviSWEET17a*, *VviHT3*, and *VviGIN1* are replaced at softening by *VviSWEET10*, *VviHT6*, and *VviTMT2*. All these genes are stopped at the arrest of phloem at 75 DAF, except for *VviSWEET15*, which remained active during shriveling (**E, F**). Graphs are expressed as surface normalized data (A, C, E) and Reads Per Kilobase per Million mapped reads (RPKM) (B, D, F). In the surface normalized graphs, each expression value was divided by the average on the 11 developmental points to reject large differences in expression among genes. Softening (S) started at an unknown date between 47 and 51 days.

Figure 6. Proton pumps and transporters genes as developmental switches. The three types of tonoplasmic proton pumps (V-PPase, V-ATPase, P-ATPase) displayed specific expression patterns during berry development. *VviPP2*, *VviVHA-A*, and *VviPH5* dramatically increased in expression with *VviHT6* at softening, 51 DAF. The peak of *VviPP1* mimicked the one of *VviHT6* during ripening (**A, B**). Only a few transporters were expressed in the green phase such as the *VviABCB17*, *VviALMT5-6*, and a still uncharacterized vacuolar membrane protein (**C, D**). Several ALMTs and TDT channels increased slightly before the ripening phases and the above mentioned changes in *VviHT6* and H⁺ pumps. Graphs are expressed as surface normalized data (A,C) and Reads Per Kilobase per Million mapped reads (RPKM) (B,D). In the surface normalized graphs, each expression value was divided by the average on the 11 developmental points to reject large differences in expression among genes. Softening (S) started at an unknown date between 47 and 51 days.

Figure 7. Fruit hormones and molecular markers of the onset of ripening. Due to the induction of *VViNCED3*, the *VviACO1/VViNCED3* (or Ethylene/ABA) balance was already impaired four days before softening, announcing the burst in *VViNCED2* expression (**A, B**). Transcription factors, such as *VviNAC26*, *VviNAC33*, *VviNAC60*, *VviSEP3*, and *VvibHLH075*, already detected at 47 days, show an obviously enhanced expression at the onset of ripening in line with their possible control of transcriptome reprogramming at this stage (**C, D**). Other putative transcription factors (*VviHB13*, *VviLBD21*, *VviPAT3*, *VviAG2*) may negatively control ripening, being enhanced during the lag phase and abruptly turned off at softening (**E, F**). Graphs are expressed as surface normalized data (A,C) and Reads

Per Kilobase per Million mapped reads (RPKM) (B,D). In the surface normalized graphs, each expression value was divided by the average on the 11 developmental points to reject large differences in expression among genes. Softening (S) started at an unknown date between 47 and 51 days.

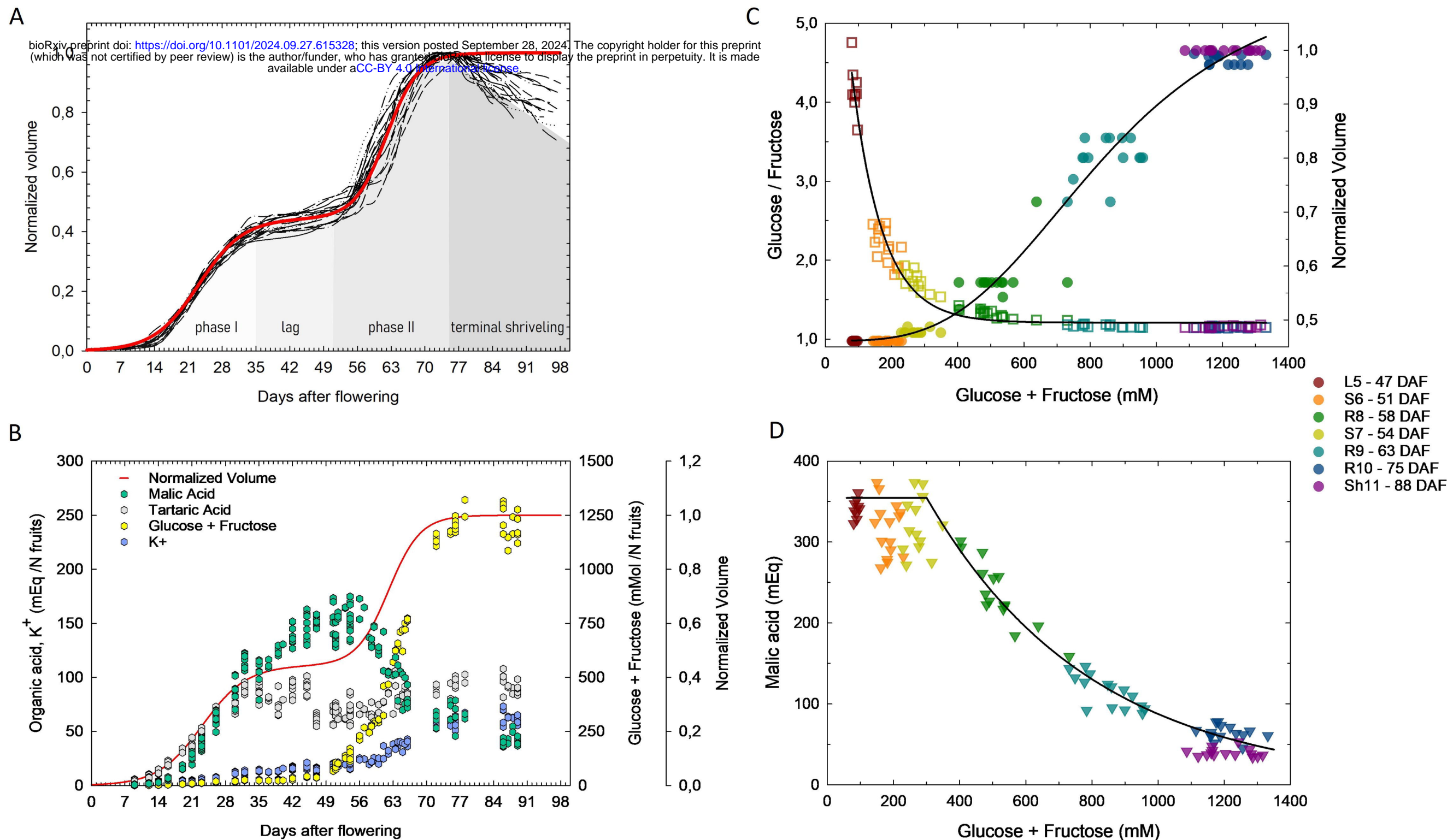


Figure 1. Growth and major osmotica in synchronized berries. A: Growth kinetics of 20 single Syrah berries (black lines). The maximal volumes were normalized, and then the first growth periods were synchronized upon shifting the individual DAF. Experimental data (black lines) were fitted to a double logistic function (red line): $v=0.44506/(1+\exp((23.14-\text{time})/4.78848)) + 0.55494/(1+\exp((62.05-\text{time})/3.31967))$, Table S3. The berry developmental phases are named according to the different shades of gray. B: Growth kinetics and accumulation of major osmotica in synchronized berries, from flowering to over-ripening. For comparative purposes, units relate to N=485 berries, yielding 1 Kg of fruit for variety Syrah at maximum volume. Green and gray dots represent malic and tartaric acids, while yellow and blue represent glucose+fructose and potassium, respectively. The detailed fluxes dataset is available in Table S4. The red line reports the fitted double sigmoidal growth curve established in Fig. 1A. C: Sugar accumulation vs. the ratio of the two hexoses. Growth resumption occurs later (at 0.4M) than softening and the onset of sugar accumulation. Squares represent the evolution of the glucose/fructose ratio, while circles show berry expansion. D: Sugar accumulation vs. malate breakdown. L5 green hard, S6-S7 green soft, R8-R9-R10-Sh11 red to black. At Sh11, the decrease in berry volume was not accompanied by visible berry wrinkling yet.

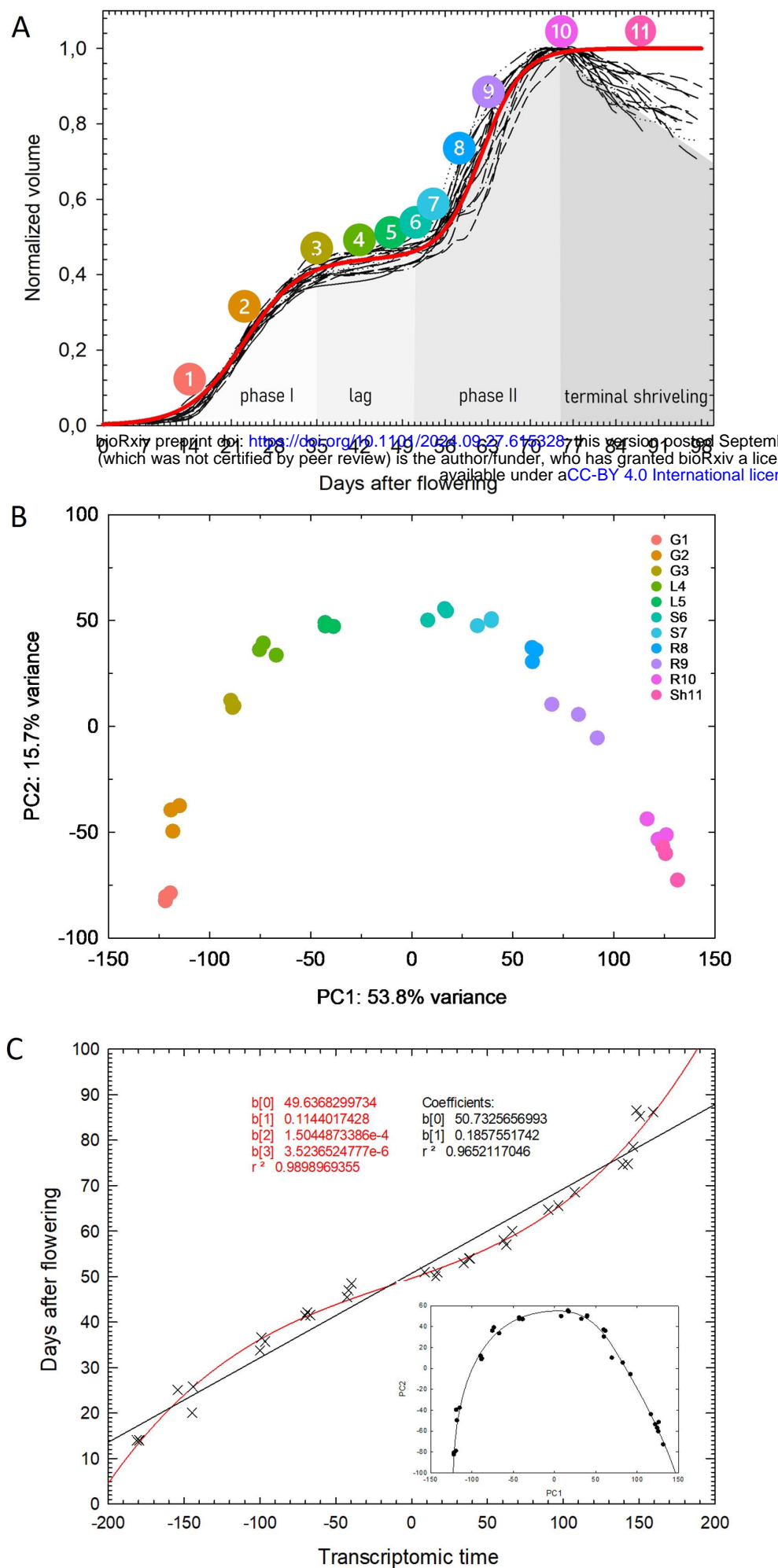


Figure 2. Transcriptomics clock overview. A: Repartition of the eleven developmental stages subjected to RNA-Sequencing according to berry growth phases. B: PCA of gene expression in 33 single berries sampled at 11 developmental stages, as color legend specifies. G: green phase, L: lag phase (green hard), S: softening (green soft), R: ripening red-purple, and Sh: shriveling. Green phase: G1 to L5, Ripening: S6 to R10, Over Ripening: Sh11. C: Transcriptomics time (red line, Table S6) is inferred from the curvilinear distance of the fitted line on the PCA (inlet).

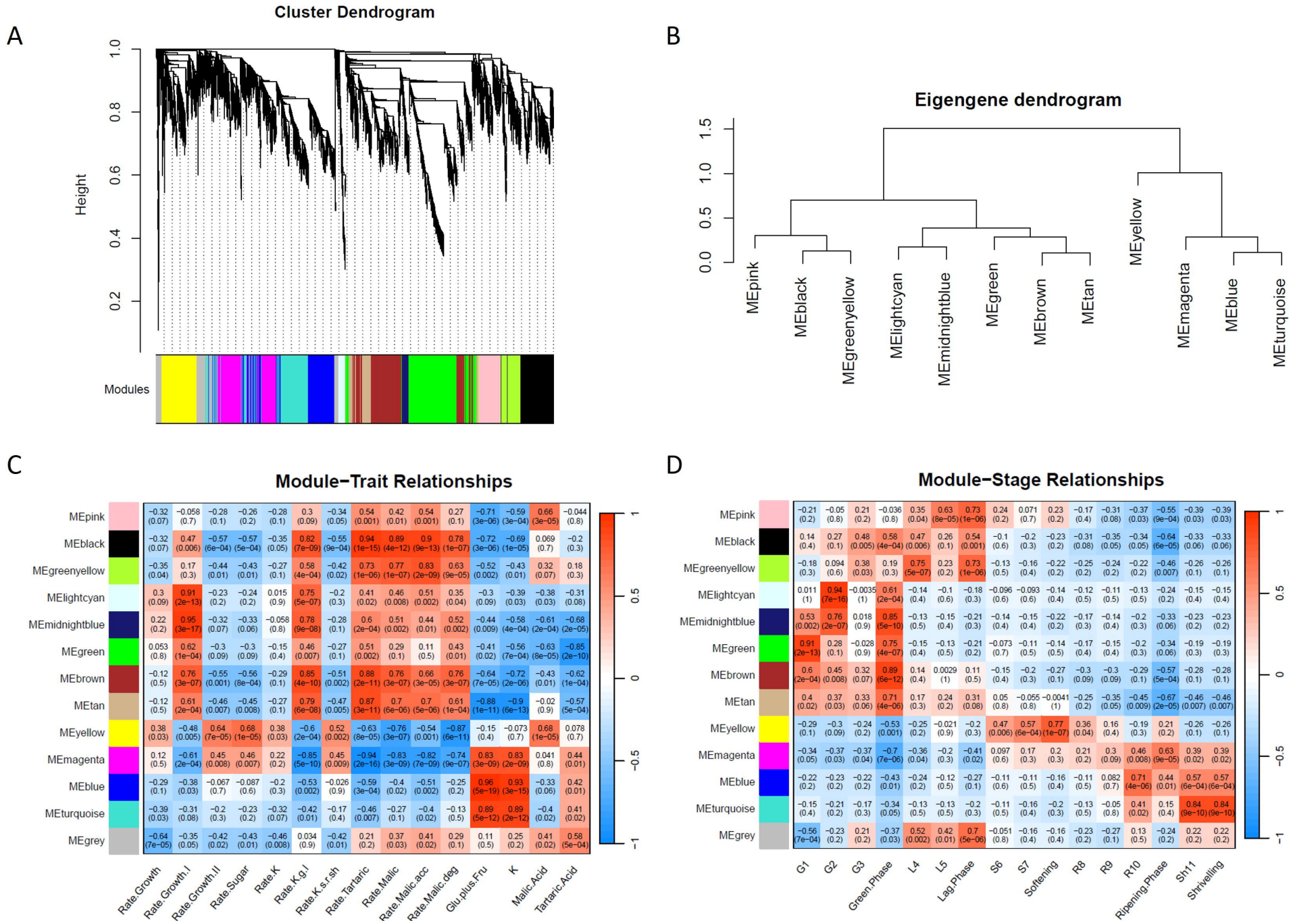


Figure 3. Weighted Gene Coexpression Analysis results. A: Hierarchical clustering tree dendrogram of 6374 time-modulated genes, with dissimilarity calculated with a soft thresholding power β of 30 based on topological overlap matrix. Each leaf (black vertical lines) corresponds to a gene. Branches of the dendrogram group together densely interconnected, highly co-expressed genes assigned 13 module colors, visible on the bottom bar. B: The Eigengene dendrogram shows the modules' distribution and interrelationships. ME = Module Eigengene. C and D: Module-Trait relationships and Module-Stage relationships, respectively. Each row corresponds to a module, and each column corresponds to a trait specified at the bottom. Numbers inside the cell describe the correlation coefficient and the associated p-value in brackets. Red and blue colors indicate a positive or negative correlation between the trait or stage and the module. Modules are listed on the right sidebar column.

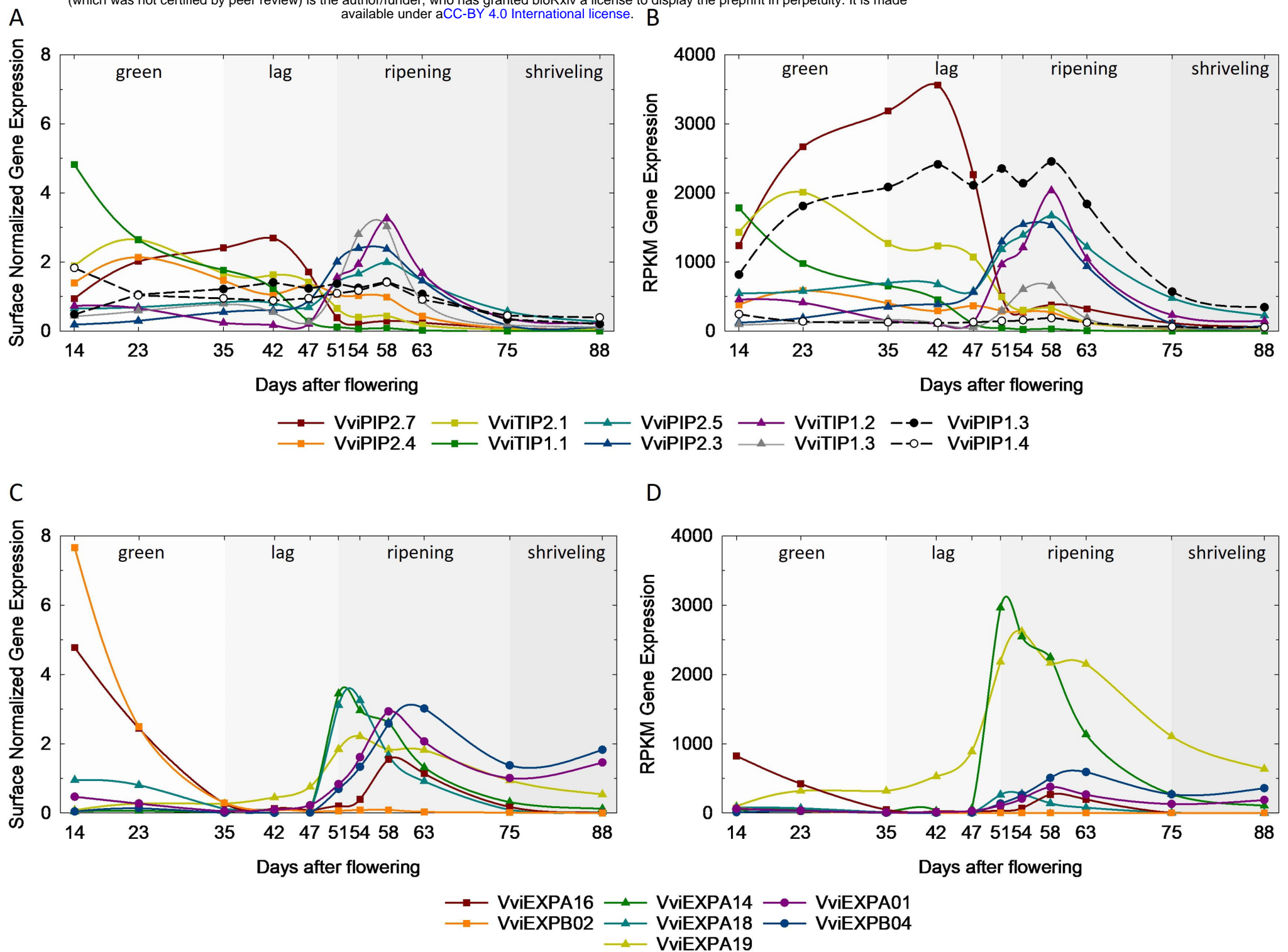


Figure 4. Fruit growth and expansion. Aquaporin (A, B) and expansin (C, D) genes active in the first period of water entry and growth phase as VviPIP2.7, VviPIP2.4, VviTIP2.1, VviTIP1.1, VviEXPA16 and VviEXPB02 and the isogenes active in the second phase of growth as VviPIP2.5, VviPIP2.3, VviTIP1.2, VviTIP1.3, VviEXPA14, VviEXPA19, and VviEXPA18. In addition, two more expansion genes were identified with an increasing trend during the shriveling time: VviEXPA01 and VviEXPB04. Lastly, two aquaporins were active in both green and ripening phases (VviPIP1.3 and VviPIP1.4). Graphs are expressed as surface normalized data (A, C) and Reads Per Kilobase per Million mapped reads (RPKM) (B, D). In the surface normalized graphs, each expression value was divided by the average on the 11 developmental points to reject large differences in expression among genes. Softening (S) started at an unknown date between 47 and 51 days.

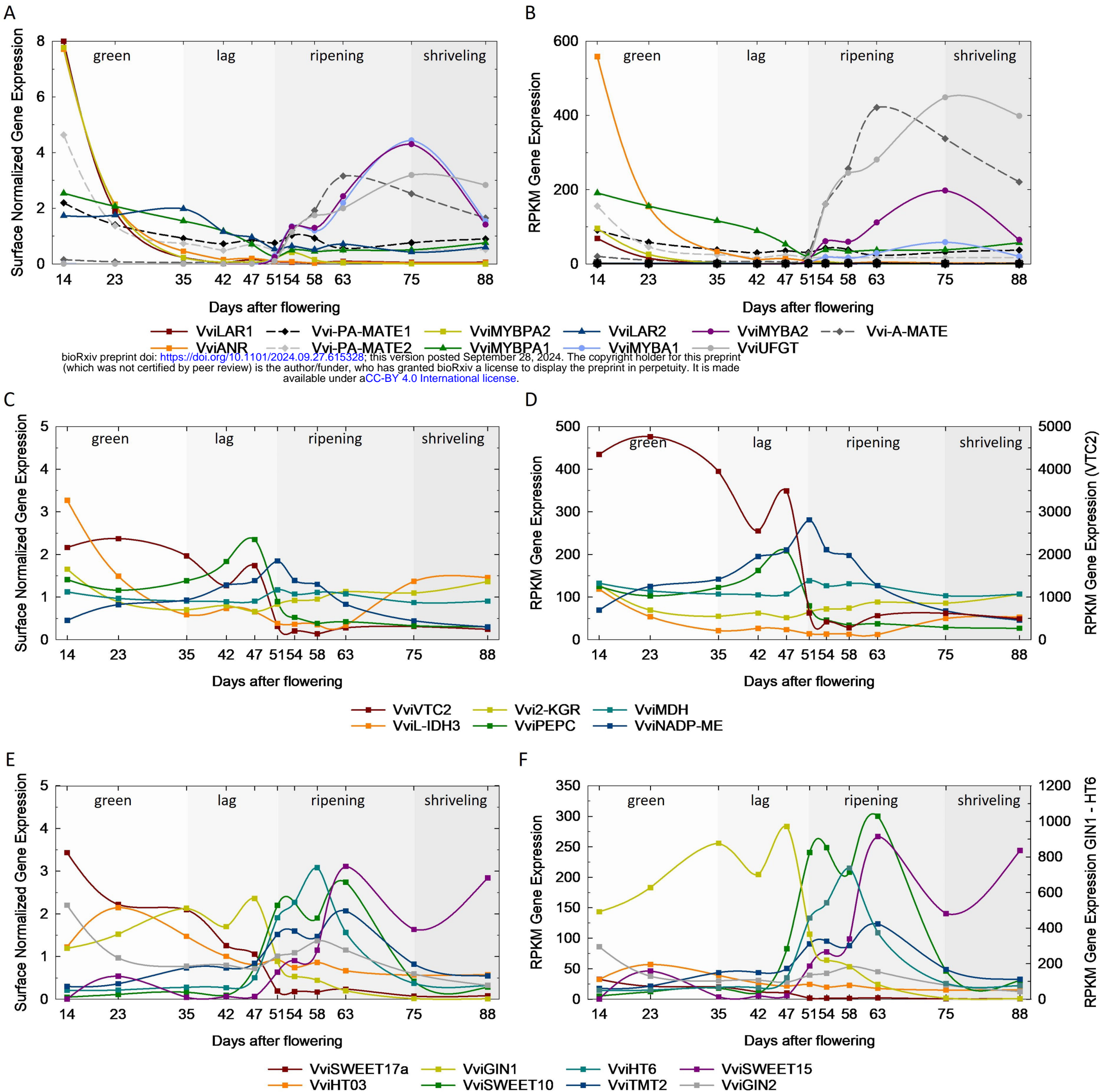
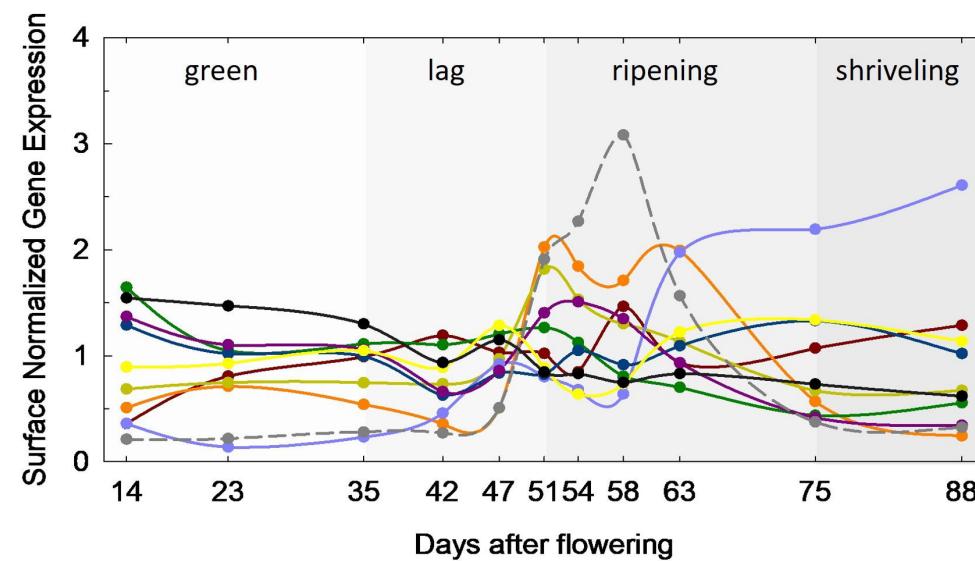
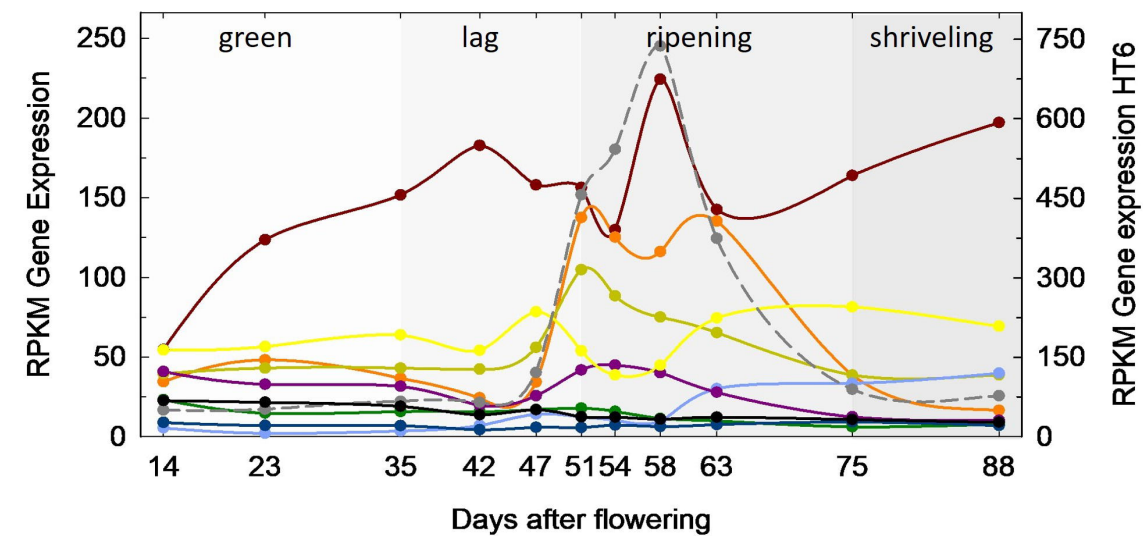


Figure 5. Key genes involved in proanthocyanidins and osmolytes biosynthesis and transport. VviLAR1, VviANR, and VviMYBPA2 genes of the flavonol pathway are expressed at the very early phase of berry development, followed by VviLAR2 and VviMYBPA1 in the green plateau; anthocyanins-related genes as VviMYBA1, VviMYBA2, and VviUFGT are activated three days after berry softening (A, B). VviVTC2, VviL-IDH, and Vvi2-KGR are known biosynthetic genes of tartaric acid, while VviPEPC, VviMDH, and VviNADP-ME are committed to malic acid synthesis and breakdown (C, D). ‘Green’ sugar transporters such as VviSWEET17a, VviHT3, and VviGIN1 are replaced at softening by VviSWEET10, VviHT6, and VviTMT2. All these genes are stopped at the arrest of phloem at 75 DAF, except for VviSWEET15, which remained active during shriveling (E, F). Graphs are expressed as surface normalized data (A, C, E) and Reads Per Kilobase per Million mapped reads (RPKM) (B, D, F). In the surface normalized graphs, each expression value was divided by the average on the 11 developmental points to reject large differences in expression among genes. Softening (S) started at an unknown date between 47 and 51 days.

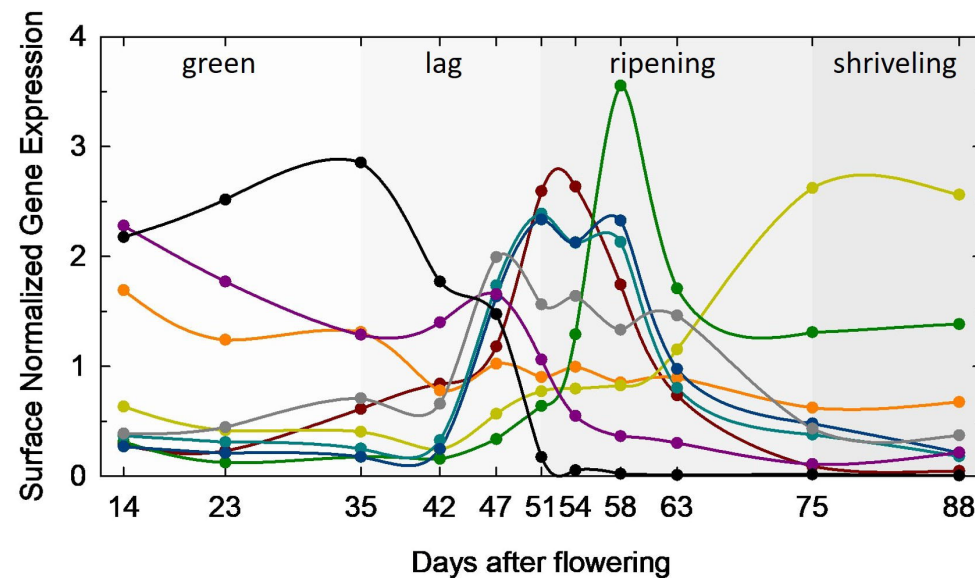
A



B



C



D

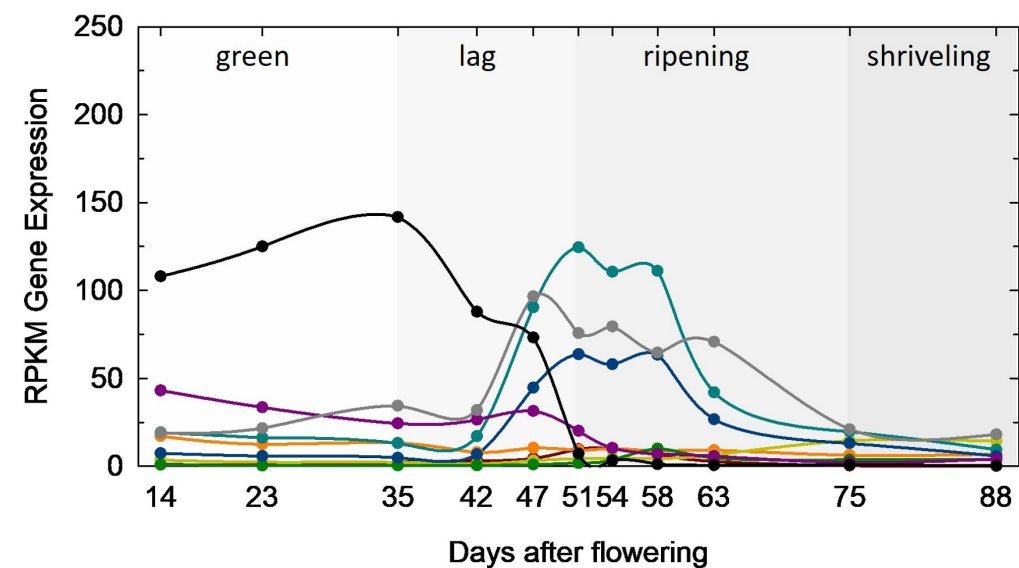


Figure 6. Proton pumps and transporters genes as developmental switches. The three types of tonoplasmic proton pumps (V-PPase, V-ATPase, P-ATPase) displayed specific expression patterns during berry development. VviPP2, VviVHA-A, and VviPH5 dramatically increased in expression with VviHT6 at softening, 51 DAF. The peak of VviPP1 mimicked the one of VviHT6 during ripening (A, B). Only a few transporters were expressed in the green phase such as the VviABCB17, VviALMT5-6, and a still uncharacterized vacuolar membrane protein (C, D). Several ALMTs and TDT channels increased slightly before the ripening phases and the above mentioned changes in VviHT6 and H⁺ pumps. Graphs are expressed as surface normalized data (A,C) and Reads Per Kilobase per Million mapped reads (RPKM) (B,D). In the surface normalized graphs, each expression value was divided by the average on the 11 developmental points to reject large differences in expression among genes. Softening (S) started at an unknown date between 47 and 51 days.

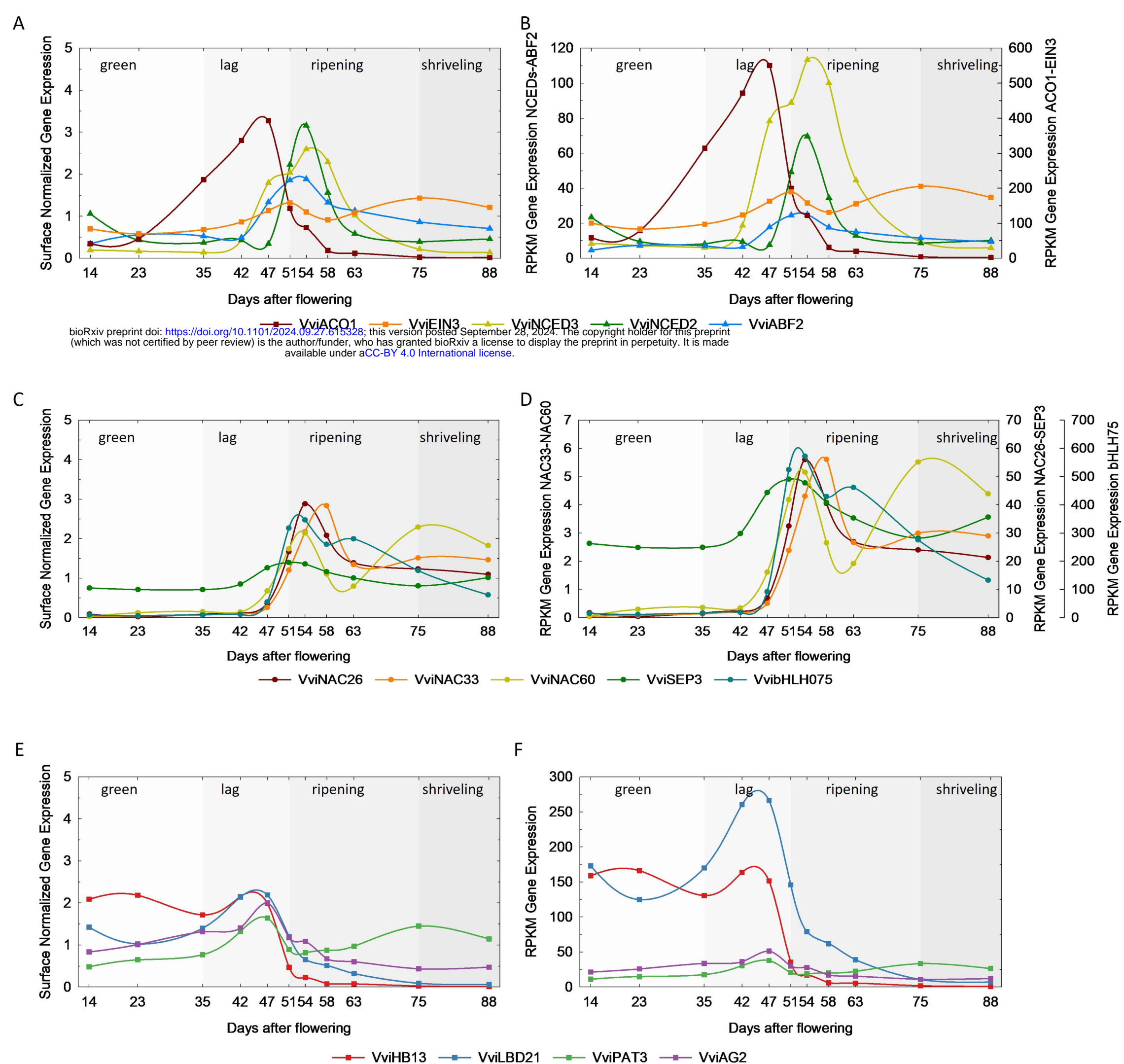


Figure 7. Fruit hormones and molecular markers of the onset of ripening. Due to the induction of VViNCED3, the VviACO1/VViNCED3 (or Ethylene/ABA) balance was already impaired four days before softening, announcing the burst in VViNCED2 expression (A, B). Transcription factors, such as VviNAC26, VviNAC33, VviNAC60, VviSEP3, and VvibHLH075, already detected at 47 days, show an obviously enhanced expression at the onset of ripening in line with their possible control of transcriptome reprogramming at this stage (C, D). Other putative transcription factors (VviHB13, VviLBD21, VviPAT3, VviAG2) may negatively control ripening, being enhanced during the lag phase and abruptly turned off at softening (E, F). Graphs are expressed as surface normalized data (A,C) and Reads Per Kilobase per Million mapped reads (RPKM) (B,D). In the surface normalized graphs, each expression value was divided by the average on the 11 developmental points to reject large differences in expression among genes. Softening (S) started at an unknown date between 47 and 51 days.