Transcriptomic analysis of temporal shifts in berry development between two grapevine cultivars of the Pinot family reveals potential genes controlling ripening time

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Abstract

Background

Grapevine cultivars of the Pinot family represent clonally propagated mutants with major phenotypic and physiological differences, such as different colour or shifted ripening time, as well as changes in important viticultural traits. Specifically, the cultivars 'Pinot Noir' (PN) and 'Pinot Noir Precoce' (PNP, early ripening) flower at the same time, but vary in the beginning of berry ripening (veraison) and, consequently, harvest time. In addition to genotype, seasonal climatic conditions (i.e. high temperatures) also affect ripening times. To reveal possible regulatory genes that affect the timing of veraison onset, we investigated differences in gene expression profiles between PN and PNP throughout berry development with a closely meshed time series and over two separate years.

Results

The difference in the duration of berry formation between PN and PNP was quantified to be approximately two weeks under the growth conditions applied, using plant material with a proven PN and PNP clonal relationship. Clusters of co-expressed genes and differentially expressed genes (DEGs) were detected which reflect the shift in the timing of veraison onset. Functional annotation of these DEGs fit to observed phenotypic and physiological changes during berry development. In total, we observed 3,342 DEGs in 2014 and 2,745 DEGs in 2017 between PN and PNP, with 1,923 DEGs across both years. Among these, 388 DEGs were identified as veraison-specific and 12 were considered as berry ripening time regulatory candidates. The expression profiles revealed two candidate genes for ripening time control which we designated *VviRTIC1* and *VviRTIC2* (VIT_210s0071g01145 and VIT_200s0366g00020, respectively). These genes likely contribute the phenotypic differences observed between PN and PNP.

Conclusions

Many of the 1,923 DEGs show highly similar expression profiles in both cultivars if the patterns are aligned according to developmental stage. In our work, putative genes differentially expressed between PNP and PN which could control ripening time as well as veraison-specific genes were identified. We point out connections of these genes to molecular events during berry development and discuss potential candidate genes which may control ripening time. Two of these candidates were observed to be differentially expressed in the early berry development phase. Several down-regulated genes during berry ripening are annotated as auxin response factors / ARFs. Conceivably, general changes in auxin signaling may cause the earlier ripening phenotype of PNP.

Key words

Vitis vinifera, Pinot Noir, Pinot Noir Precoce, grapevine, berry ripening, fruit development, differential gene expression, transcriptome profiling, ripening time control, veraison

63 **Background**

64 *Vitis vinifera subsp. vinifera* (grapevine) belongs to the family *Vitaceae*. With 6,000 to 11,000
65 cultivars, it is one of the most important perennial crops worldwide [1]. Grapevine fruit
66 development can be divided into two physiological phases, berry formation and berry
67 ripening. Veraison refers to the transition from berry formation to berry ripening, and each
68 of the two phases is represented by a sigmoidal growth curve of development [2]. The
69 progress through development is described by stages referred to as "BBCH stages" (acronym
70 derived from the names of the coordinating institutions involved in stage definition) that
71 have been defined for several crops including grapevine [3, 4]. The first physiological phase is
72 described as berry formation (berry initiation and growth with cell divisions) and lasts from
73 the end of flowering (BBCH71) until ~60 days later when the majority of berries are touching
74 each other (BBCH79). The developmental stage of veraison (BBCH81) is the end of berry
75 formation and the start of berry ripening [2]. Phenotypically, veraison is the developmental
76 switch when the berries start to soften, accompanied by the onset of accumulation of
77 phenylpropanoids. In red grapevine cultivars, veraison is also indicated by a colour change of
78 the berries that is caused by the beginning of accumulation of anthocyanins, a major class of
79 phenylpropanoids. Members of the well-studied protein superfamily of R2R3-MYB
80 transcription factors (TFs) are considered to be mainly accountable for controlling
81 anthocyanin accumulation [5-7]. Berry ripening starts at veraison and continues until harvest
82 (BBCH89), this phase includes cell enlargement, sugar accumulation and acidity decline.
83 Timing of veraison has also been studied at the level of genetic loci and genomic regions that
84 control this trait. Since it is a quantitative trait influenced by several to many genetic loci,
85 quantitative trait locus (QTL) analyses have been performed. These studies detected a major
86 QTL for timing of the onset of veraison on chromosome 16, combined with a minor QTL on

87 chromosome 18 [8]. By integrating a number of QTL studies, several meta-QTLs connected
88 to genetic control of veraison time were detected, with the most relevant located on
89 chromosome 14, 16, and 18 [9].
90 Anthropogenic climate change is resulting in successively earlier ripening of grapes with a
91 significant impact on berry quality and consequently the expected flavours of a desired wine
92 style [10]. In addition, the time of veraison and harvest of a given cultivar may differ greatly,
93 driven by regional and/or year-specific differences in weather conditions. Obviously, this
94 calls for a better molecular understanding of the control of ripening time in grapevine.
95 Comparison of different grapevine cultivars grown at the same environmental conditions
96 often uncovers differences in the duration of berry formation, timing of veraison, duration of
97 berry ripening, and ripening time in general. However, the underlying genetic factors are
98 mostly unknown. Previous studies have elucidated how ripening time is affected by internal
99 and external factors. For example, the effect of phytohormones on berry ripening has been
100 widely studied [1]. In general, fruit growth is discussed to be controlled by several
101 phytohormones which play essential roles to trigger or delay ripening processes [11].
102 Grapevine is a non-climacteric fruit and effects of abscisic acid (ABA) have been investigated
103 in many studies as ABA is considered to trigger ripening [12-14]. Furthermore, it was shown
104 that ABA is involved in controlling leaf senescence [15], responses to drought [16] and
105 pathogen defense [17]. In grapevine, although not as central as in climacteric fruits like
106 tomato (*Solanum lycopersicum*), the phytohormone ethylene is involved in the control of
107 berry ripening [1, 13, 18, 19], while auxin has been shown to induce a delay of ripening [20,
108 21].
109 Fruit development of both, dry and fleshy fruits, has been studied very intensively for the
110 obvious reason that fruits are central to human nutrition [22, 23]. The main model system

111 for studies on fleshy fruits is tomato, because of established genetics and molecular biology,
112 access to mutants, and well advanced transgenic approaches to gene function identification
113 [24, 25]. Berry development of grapevines has also been studied intensively [1, 26] and often
114 at the level of the transcriptome. In quite some of the studies, predominantly late berry
115 development stages were sampled to bring the development stage of veraison into the focus
116 [9, 27-29]. In addition, whole berry development was studied with coarse time point
117 distribution [30-34].
118 To monitor gene expression changes at a high resolution throughout grapevine berry
119 development, starting from flowering until berries are matured, we sampled a
120 comprehensive time series from two Pinot cultivars across two years. The samples were
121 collected from the grapevine cultivar 'Pinot Noir' (PN) and the comparably earlier ripening
122 cultivar 'Pinot Noir Precoce' (PNP) that is expected to be closely related to PN. The cultivar
123 PNP is listed in the *Vitis* International Variety Catalogue (*V*IVC; [35]) and described to flower
124 at the same time as PN but to reach veraison significantly earlier than PN [36]. Quantitative
125 data for transcript levels, interpreted as values for gene expression, were generated by RNA-
126 Seq. We studied the general course of gene expression patterns throughout berry
127 development in both years and cultivars, and identified a number of differentially expressed
128 genes (DGEs) between PN and PNP prior to veraison. These DEGs can be considered as
129 important candidates for either delaying or pushing forward berry development. Our main
130 aim was the identification of genes controlling the speed of development, to offer an entry
131 point into characterization of the relevant molecular functions in grapevine, and to facilitate
132 future breeding strategies that address traits relevant to, and affected by, climate change.
133
134

Results

Phenotypical comparison between two Pinot cultivars

To study ripening shifts, we used samples of two closely related grapevine cultivars. The cultivar PNP is an earlier ripening clonal variant of its ancestor PN. Clonal relation of PN and PNP was confirmed by a set of 24 SSR markers that all displayed the identical allele status for both cultivars (Additional file 1: Table S1). To confirm and validate the phenotypic differences between PN and PNP, detailed BBCH developmental stages were determined and documented (Figure 1). PN and PNP display similar phenotypic properties during development and flower (BBCH65) at the same time. However, veraison (BBCH81) is shifted to ~2 weeks earlier for PNP, and similar shifts were observed in four different years (Table 1). In addition, Figure 1A shows an overview across the time points at which samples were taken. The phenotypic differences between PN and PNP are illustrated in images of developing berries taken between onset of berry formation and veraison (Figure 1B and Additional file 1: Table S2 and Table S3). Veraison (BBCH81) is visible as the onset of anthocyanin accumulation and is detected ~2 weeks later in PN compared to PNP.

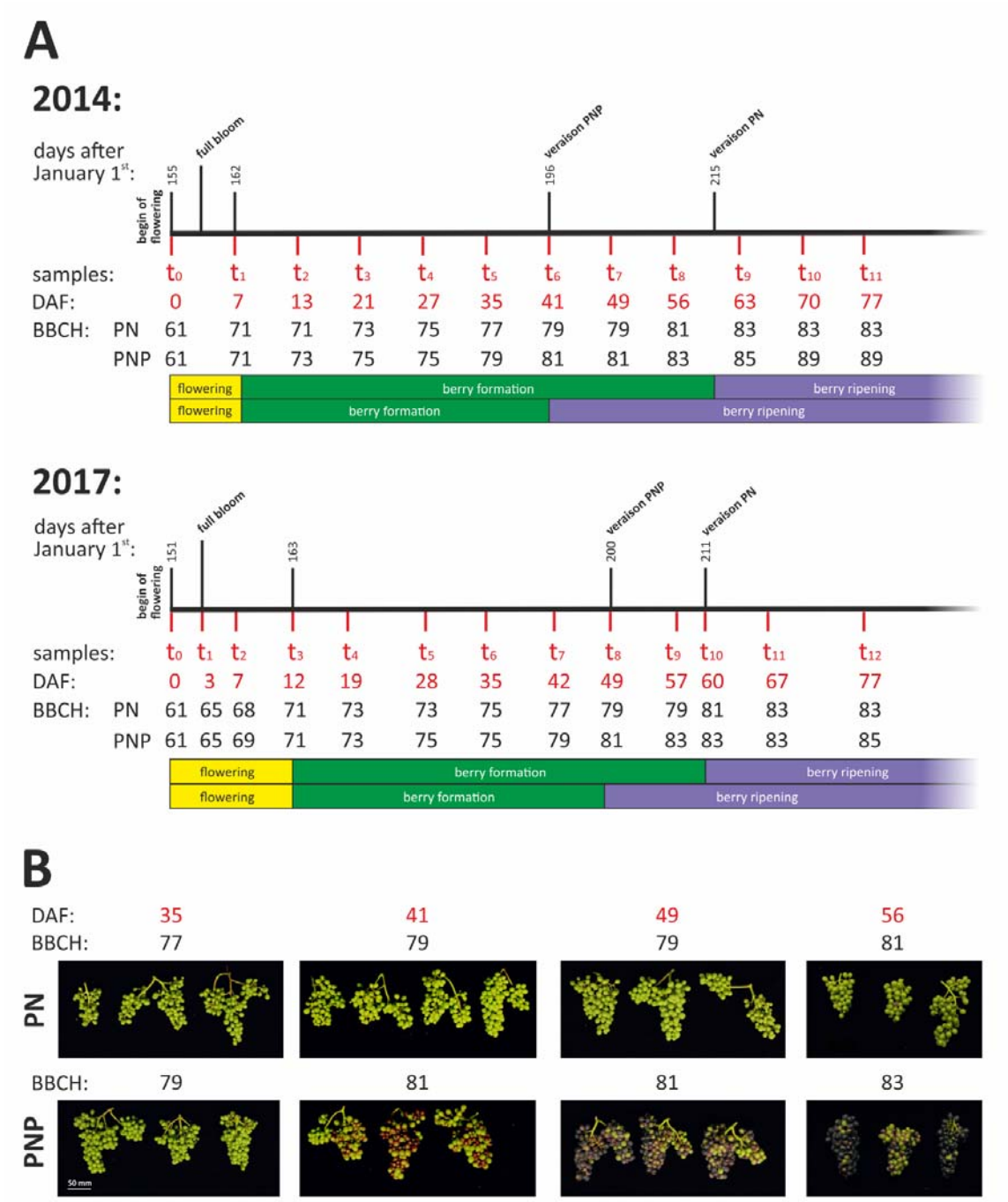


Figure 1: Phenotypical observations and sampling scheme. (A) Sampling time points and days after onset of flowering (DAF) are indicated in red. The developmental stage observed is shown in the BBCH stages [3, 4]. DAF zero (0) is set at BBCH61 (onset of flowering, 10% of flowerhoods fallen [3]). Berry development is depicted schematically and categorized into the phases flowering (yellow), berry formation (green), and berry ripening (purple) for both cultivars. The junction between green and purple indicates veraison (BBCH81). To orient for

158 time of year, numbered days after January 1st are shown. (B) Images of grape bunches and
159 developing berries taken in 2014 are shown to document the differences between PN and
160 PNP. Images were taken 35, 41, 49 and 56 DAF. Scale bar: 50 mm.
161

162 **Table 1**: Observed flowering- and berry development shifts between the cultivars PNP and
163 PN in 2014, 2015, 2016 and 2017 at the Geilweilerhof vineyards, Siebeldingen, Germany (in
164 days after January 1st).

Year	Cultivar	Start of flowering (BBCH61)	End of flowering period / Start of berry formation (BBCH71)	End of berry formation / veraison (BBCH81)	Flowering time [Δ days]	Berry formation time [Δ days]
2014	PNP	155	162	196	0	19
2014	PN	155	162	215		
2015	PNP	159	166	201	7	14
2015	PN	159	173	222		
2016	PNP	171	180	215	0	14
2016	PN	171	180	229		
2017	PNP	151	163	200	0	11
2017	PN	151	163	211		

165

166 **Global view of gene expression patterns**

167 We harvested triplicate samples in 2014 and 2017 from flowering until after veraison (for
168 time points see Figure 1 and Additional file 1: Tables S2 and S3), individual harvests are
169 referred to below as subsamples, and analyzed them by RNA-Seq. After preprocessing the
170 raw data (see Methods), reads derived from each subsample were mapped to the reference
171 sequence from PN40024 and analyzed with respect to the CRIBI V2.1 annotation dataset. For
172 2014, approximately 19.7 million reads per subsample were obtained from each of the 72
173 libraries. An overall alignment rate of 79 % to the grape reference genome sequence was
174 reached. For 2017, approximately 43.5 million reads per subsample were obtained from
175 each of the 78 libraries. From these, an overall alignment rate of 92 % to the reference was

176 calculated. Expression values were initially detected as Transcripts Per Kilobase Million
177 (TPM) and averaged over the three subsamples for each sample. Considering both years
178 separately, a total of 28,692 genes were detected as expressed in both cultivars and in both
179 years. In contrast, 2,152 CRIBI V2.1 genes were found to be not expressed.
180 The correlation between gene expression data, determined as TPM values per sample, of the
181 datasets from both years over all genes was $r = 0.5095$ (Pearson correlation coefficient) for
182 PN and $r = 0.6557$ for PNP, respectively. For PN and PNP, 10,205 and 16,226 genes,
183 respectively, expression values were significantly correlated (p-value < 0.05) between the
184 years 2014 and 2017. A list of the correlation strength of the eight time points with the same
185 BBCH stage is provided in Additional file 1: Table S4.
186 To visualize global trends and similarity of the gene expression values obtained from all
187 subsamples, a Principal Component Analysis (PCA) of both years was performed with
188 keeping the subsamples separate. The first component PC1 explains 57% of the variance,
189 while the second component PC2 explains 17% (Figure 2). Almost all data points of the
190 subsamples (triplicates within a sample) from both years cluster near to each other. The data
191 follow a track of time in a nearly consecutive and continuous way. Main actors, which
192 influence most of the variance in the data, were genes related to cell wall modification,
193 secondary metabolism, wounding-response and hormone signaling. The top 500 genes
194 responsible for most of the variance in PC1 and PC2 are listed in Additional file 1: Table S5
195 and Table S6, together with functional information for each listed gene from
196 MapMan/Mercator and RefSeq.
197

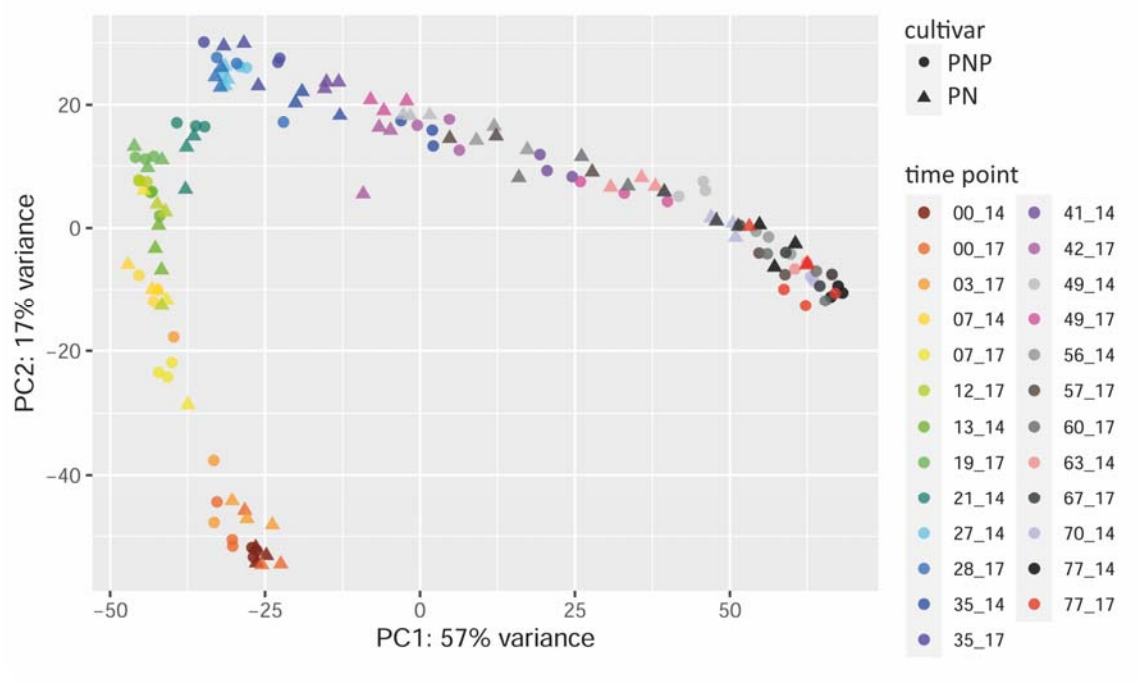


Figure 2: Principle Component Analysis (PCA) of gene expression values from all subsamples. Each data point represents a single subsample of the triplicates for each time point of both years (2014 and 2017 as indicated by [DAF]_14 and [DAF]_17 with the colour code) and for both cultivars (PN as triangles, PNP as circles).

Cluster analysis for identification of co-expressed genes

Gene expression time series profiles combined data from the subsamples/triplicates for each time point across two years for both culrivars. Expression profiles were compared using the clustering tool CLUST. The goal was the characterization of similarity and/or differences in gene expression among years and cultivars throughout berry development. Over all four datasets, 13 PN/PNP clusters of genes with similar gene expression patterns (C1-C13) were obtained (Additional file 2: Figure S1A). In these clusters, 3,316 (12.2 %) of the 27,139 genes expressed during berry development were found co-expressed among both years and cultivars. It should be noted that CLUST uses criteria to define expressed and not expressed

213 genes that differ from the ones applied above (see Methods). The observed expression
214 profiles differ clearly between the clusters, which was in part the result of the restricted
215 number of clusters that CLUST extracts. Manual inspection of the clusters revealed little
216 deviation of individual gene expression profiles within each individual cluster for a given year
217 or cultivar. All cluster gene memberships, also those for the additional cultivar-specific
218 cluster analyses (see below), are available in Additional file 1: Table S7-S9.
219 The PN/PNP clusters C2, C5, C6 and C12 (C12_PN/PNP selected as example, see Figure 3)
220 reveal a small but detectable difference in the gene expression profile between both
221 sampled years, but are almost identical for both cultivars. The PN/PNP clusters C1, C7 and
222 C11 (C1_PN/PNP selected as example, see Figure 3) show similar expression profiles over the
223 two years, but stand out by shifted expression peaks that distinguish PN and PNP.
224 To characterize the clusters with respect to potential functions of the co-expressed genes
225 included in a given cluster, GO term enrichment for biological processes was calculated. The
226 full list of enriched GO terms for all clusters is listed in Additional file 1: Table S10-S12. Two
227 examples for GO terms appearing with highly significant incidence were 'response to
228 oxidative stress' in cluster C11_PN/PNP (term GO:0051276) and 'regulation of defense
229 response' in cluster C5_PN/PNP (term GO:0031347).
230 Two additional cluster analyses were performed, one for the PN data from both years
231 (Additional file 2: Figure S1B) and one for the PNP data from both years (Additional file 2:
232 Figure S1C). These analyses revealed a high abundance of genes from the expansin gene
233 family with similar expression profiles in clusters C0_PNP and C6_PN. The cluster C16_PN
234 was found to have a highly significant enrichment for 'vegetative to reproductive phase
235 transition' (GO:0010228). Cluster-gene memberships for the cultivar-specific clustering are

available in Additional file 1: Table S8-9, and the corresponding GO term enrichment is summarized in Additional file 1: Table S11-12.

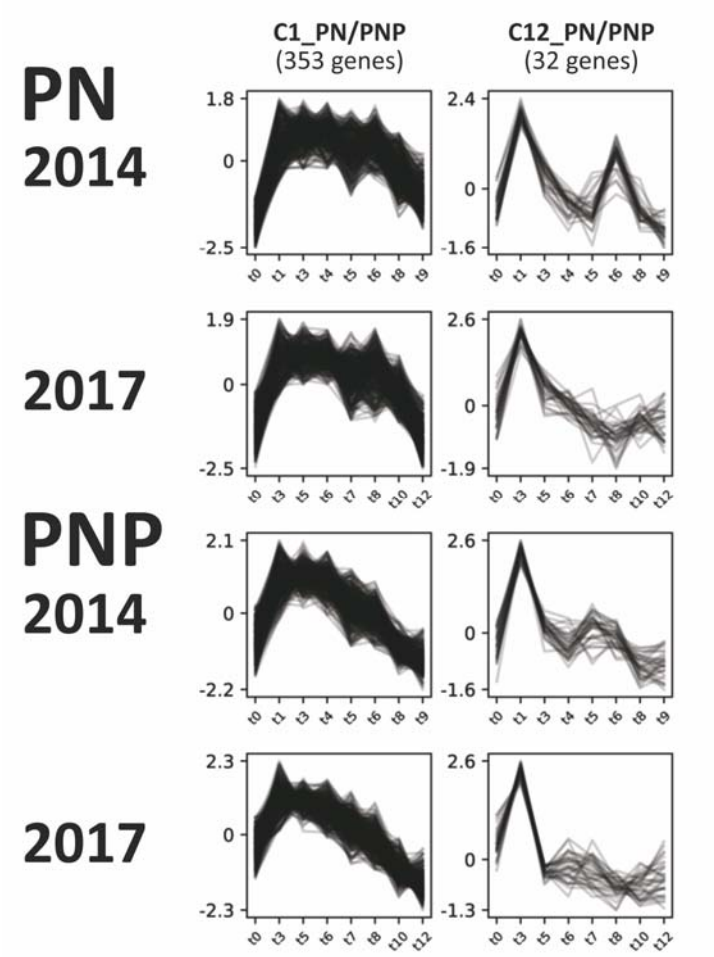


Figure 3: Two selected gene expression profile clusters with either a cultivar-specific difference (C1_PN/PNP) or a weather/field condition-specific difference (C12_PN/PNP) after clustering all data (both cultivars and both years). Strength of gene expression (quantile normalization) was plotted over the time course of berry development. Sampling time points are detailed in Figure 1 and were restricted to those eight equivalent time points at which the cultivars display the same BBCH stage (Additional file 1: Table S4). For all PN/PNP clusters see Additional file 2: Figure S1A. IDs of genes that make up the clusters are listed in Additional file 1: Table S7.

249 **Analyses of differentially expressed genes**

250 The gene expression time series throughout berry development was analyzed for
251 differentially expressed genes (DEGs) between the two cultivars PN and PNP with DESeq2.
252 Genes with significantly differential expression were selected by using the filters adjusted
253 p-value (PADJ) < 0.05 and log2fold change (LFC) > 2. The results are summarized in Table 2
254 and are detailed at the gene level per time point compared in Additional file 1: Table S13.

255

256 **Table 2:** Filtering steps applied for selecting DEGs, and the number of DEGs that were carried
257 on after each selection step. For details see Methods.

	PN/PNP 2014 [DEGs]	**PN/PNP 2017 [DEGs]**
adjusted p-value (PADJ) < 0.05: (counted over all sample pairs)	8,206	4,419
log2fold change (LFC) > 2: (counted over all sample pairs)	6,629	4,298
down- / up-regulated (in PNP vs. PN**):**	3,293 / 3,336	2,130 / 2,168
unique: (non-redundant within time series)	3,342	2,745
intersection: (detected in both years)	1,923	
excluded due to intersection:	*1,419*	*822*
veraison-specific genes: (detected within BBCH79-81 of PNP)	388	
potentially regulatory: (detected within BBCH61-79 of PNP)	12	

258

259 In total, 8,206 and 4,419 DEGs were identified with PADJ greater than 0.05 for 2014 and
260 2017, respectively. Almost twice as many DEGs were initially detected in 2014 samples
261 compared to 2017. By applying the filter for an at least 2-fold difference in expression level
262 (LFC > 2), the number of significant DEGs decreased, mainly for the PN/PNP time series from

263 2014. Only few DEGs between PN and PNP were observed during flowering (BBCH61 to 69)
264 at the beginning of both time series (see Figure 4). Within the berry formation phase
265 (BBCH71 to 79), the number of DEGs detected increased towards veraison (BBCH81) as both
266 genotypes increasingly varied in physiological stages. The highest number of DEGs was
267 observed in parallel to the time-shifted veraison of PNP relative to PN. This time point was
268 also the most phenotypically different between the two cultivars (see Figures 1 and 4). A set
269 of veraison-specific genes was defined by selecting the DEGs from time points DAF35 and
270 DAF41 from 2014 that were also observed to coincide with this phenotype at DAF42 and
271 DAF49 in the 2017 gene expression data. These criteria identified 388 veraison-specific
272 DEGs. This set of 388 genes was compared to results from similar studies and found to be in
273 agreement (e.g. 81% [27] and 52% [28]; IDs of the 388 genes, the genes that match results
274 from the other studies and their functional annotation, are included in Additional file 1:
275 Table S14). During the subsequent phase of berry ripening (BBCH81 to 89, after around
276 DAF56 in 2014 and around DAF60 in 2017), the number of DEGs detected decreased.
277 We developed a visualization for the numbers of DEGs detected and the changes with
278 respect to which genes are newly appearing as differentially expressed at a given time point
279 (sample pair PN/PNP) in the time series (Figure 4). Groups of newly appearing DEGs are
280 containing only few genes early in berry formation, while numbers increase at veraison of
281 PNP. After veraison of PN, the number of DEGs decreased. If DEGs appearing in several time
282 points are counted only once, 3,342 and 2,745 unique DEGs (different genes) are detected
283 from 2014 and 2017, respectively (compare Table 2).
284

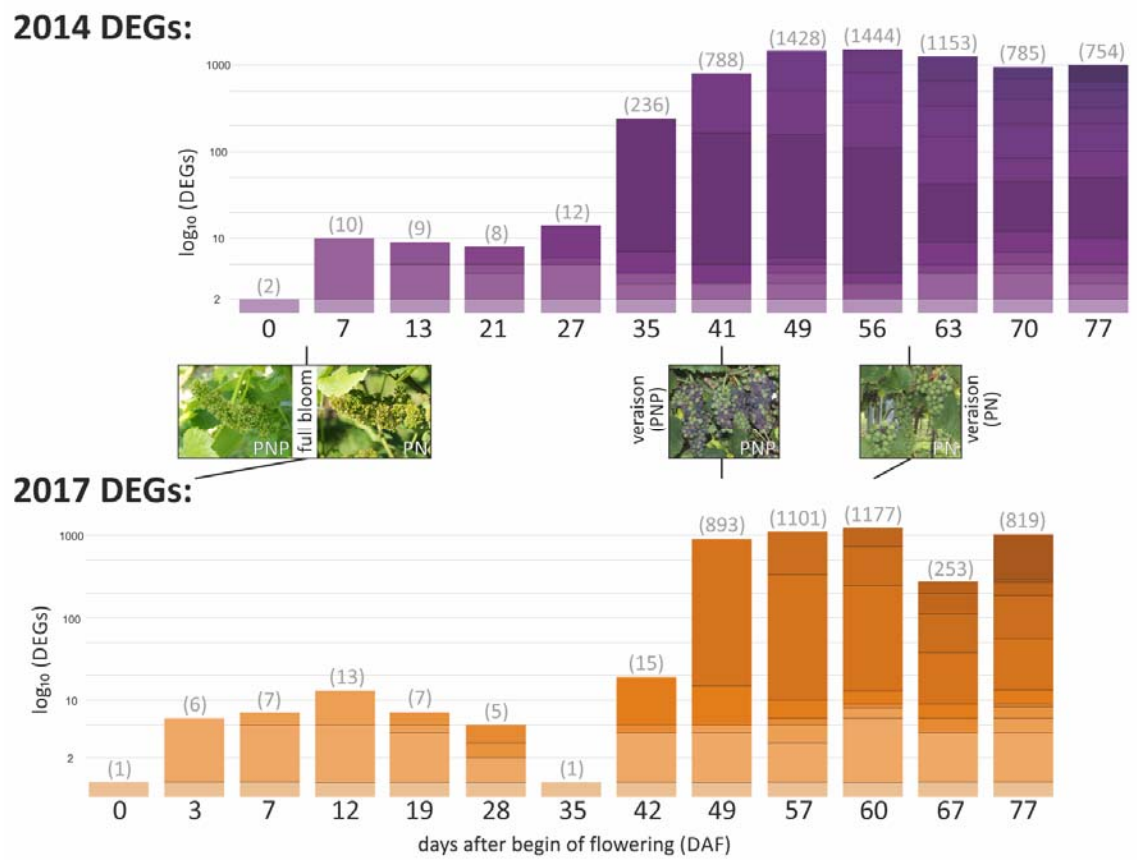


Figure 4: Visualization of the number of DEGs detected between PN and PNP in a logarithmic scale (log_{10}). Results for 2014 are shown in purple, those for 2017 in orange. The time series from the two years were aligned at veraison of PNP; the timeline is given as days after onset of flowering (DAF). DEGs are counted for a pair of PN/PNP samples for each time point individually, the number above each column mentions the number of DEGs detected at the respective time point. Groups of newly appearing DEGs relative to an earlier time point are indicated by a new colour shade in the column (bar) for each time point. For members of a given group of DEGs, the attributed colour shade is kept for the subsequent time points (columns/samples). The pictures between the two column series display BBCH65 (full bloom, 50% of flowerhoods fallen [3]) and BBCH81 (veraison) of PNP and PN.

To further increase the reliability, reproducibility, and relevance of the selected DEGs, the intersection between the DEGs identified in the two years studied was computed. In total, 1,923 unique DEGs were obtained (Table 2). To reveal DEGs potentially involved in the

300 control of timing of ripening, i.e. genes that might be involved in the trait that mainly
301 distinguishes PN and PNP, only intersecting DEGs which appeared at time points before
302 veraison in PNP were picked. This resulted in a list of 12 DEGs that may control ripening
303 time. It should be noted that these putative regulatory DEGs are supposed to be relevant
304 before the set of veraison-specific genes implements the phenotypic changes at veraison.
305 The full list of DEGs, their identity and annotation information as well as their fit to the
306 selection criteria on the way from all (raw) DEGs to potentially regulatory DEGs are detailed
307 in Additional file 1: Table S13. IDs of the 12 putative ripening time control genes, the genes
308 that match results from related studies (7 DEGs [27], 4 DEGs [34] and 3 DEGs [28]) and their
309 functional annotation are included in Additional file 1: Table S14, the most relevant data are
310 summarized in Table 3.
311
312 **Functional classification of DEGs**
313 To complement the gene lists with functional information from grapevine that might
314 potentially be informative for berry development, the 1,923 intersecting DEGs were
315 analyzed with respect to enrichment of genes that have been assigned to biological
316 pathways already established for grapevine (see Methods). For 46 of the 247 defined
317 grapevine pathways, significant enrichment (permuted p-value <0.1) was detected. The most
318 reliable predictions (permuted p-value <0.001) for pathways that might be relevant were
319 photosynthesis antenna proteins (vv10196; 9 DEGs); nitrogen metabolism (vv10910, 19
320 DEGs); phenylpropanoid biosynthesis (vv10940, 66 DEGs), tyrosine metabolism (vv10350, 33
321 DEGs); transport electron carriers (vv50105, 18 DEGs); phenylalanine metabolism (vv10360,
322 33 DEGs); brassinosteroid biosynthesis (vv10905, 8 DEGs) and flavonoid biosynthesis
323 (vv10941, 30 DEGs). The enrichment results are provided in (Additional file 1: Table S15). The

324 same analysis was also carried out for the 12 putative ripening time control (Additional file 1:
325 Table S16) and the 388 veraison-specific DEGs (Additional file 1: Table S17).
326 A check of the 1,923 intersecting DEGs revealed that 141 TF genes are included. Of these, 48
327 DEGs were clearly up- and 93 down-regulated at their first appearance in the time series.
328 The full list of TF encoding genes that were higher expressed in PNP (up-regulated), or lower
329 expressed in PNP (down-regulated), compared to PN, is shown in Additional file 1: Table S18.
330 For a more detailed view on the expression patterns of selected TF encoding genes, we
331 generated for the TF gene family with the highest abundance among the 141 TF genes,
332 namely the R2R3-MYB-type TFs with 22 cases in the MapMan functional assignment, an
333 expression heatmap (Additional file 2: Figure S2). As a result, *VviMYB24*
334 (VIT_214s0066g01090), which is related to At3g27810/*AtMYB21*, At5g40350/*AtMYB24* and
335 At3g01530/*AtMYB57* according to TAIR/PhyloGenes, was identified as an early appearing
336 DEG that showed its highest expression level at flowering (BBCH61). Prominent *R2R3-MYB*
337 genes known to be relevant for anthocyanin accumulation like *VviMYBA1*
338 (VIT_202s0033g00410), *VviMYBA2* (VIT_202s0033g00390), *VviMYBA3*
339 (VIT_202s0033g00450) and *VviMYBA8* (VIT_202s0033g00380) were detected as expressed
340 starting from veraison (BBCH81) in both cultivars and with a time shift towards earlier
341 expression in PNP. An additional *R2R3-MYB* gene with a similar expression pattern is
342 *VviMYB15* (VIT_205s0049g01020). Other *R2R3-MYB* genes are expressed early during berry
343 formation, these include *VviMYBF1* (VIT_207s0005g01210, related to
344 At2g47460/*AtMYB12*/*AtPFG1*) as well as *VviMYBPA5* (VIT_209s0002g01400) and *VviMYBPA7*
345 (VIT_204s0008g01800, both related to At5g35550/*AtMYB123*/*AtTT2*). According to their
346 related expression patterns visualized in the heatmaps (Additional file 2: Figure S2), the

347 *R2R3-MYB* genes fall into three groups that roughly fit to the three phases marked in Figure
348 1B, namely flowering, berry formation, and berry ripening (see discussion).
349
350 **Putative candidates for ripening time control genes**
351 As mentioned above, DEGs detected in both years at time points before veraison of PNP
352 were selected and considered as putative genes that control ripening time (Table 3,
353 Additional file 1: Table S13). The VitisNet enrichment analyses performed for these 12
354 candidates resulted in 2 pathways that showed significant (permuted p-value < 0.05)
355 enrichment with two genes in the pathway: auxin signaling (vv30003 with *VviEXPA5*
356 (VIT_206s0004g00070) and *VviEXPA14* (VIT_213s0067g02930)) and cell wall (vv40006 with
357 *VviPL1* (VIT_205s0051g00590) and *VviGRIP28* (VIT_216s0022g00960)); see Additional file 1:
358 Table S16).
359 A detailed check of the data presented in Figure 4, together with results for these putatively
360 ripening time control DEGs, resulted in the identification of two DEGs that stand out from
361 the whole list of DEGs. Both genes almost completely lack expression in the early ripening
362 cultivar PNP while there is clear expression in PN. Therefore, these two genes were detected
363 as DEGs throughout the whole time series in both years. The first of the two, designated
364 *VviRTIC1* for "Ripening Time Control" (VIT_210s0071g01145, encoding a protein similar to
365 "protein of unknown function DUF789"), is expressed during flowering (BBCH61 - 65) and is
366 more or less continuously down-regulated over time in PN (Figure 5A). The second of the
367 two, designated *VviRTIC2* (VIT_200s0366g00020, encoding a protein similar to "cysteine-rich
368 receptor-like protein kinase"), displays expression in PN during berry formation as well as
369 during berry ripening with a peak before veraison in 2017 (Figure 5B). The expression

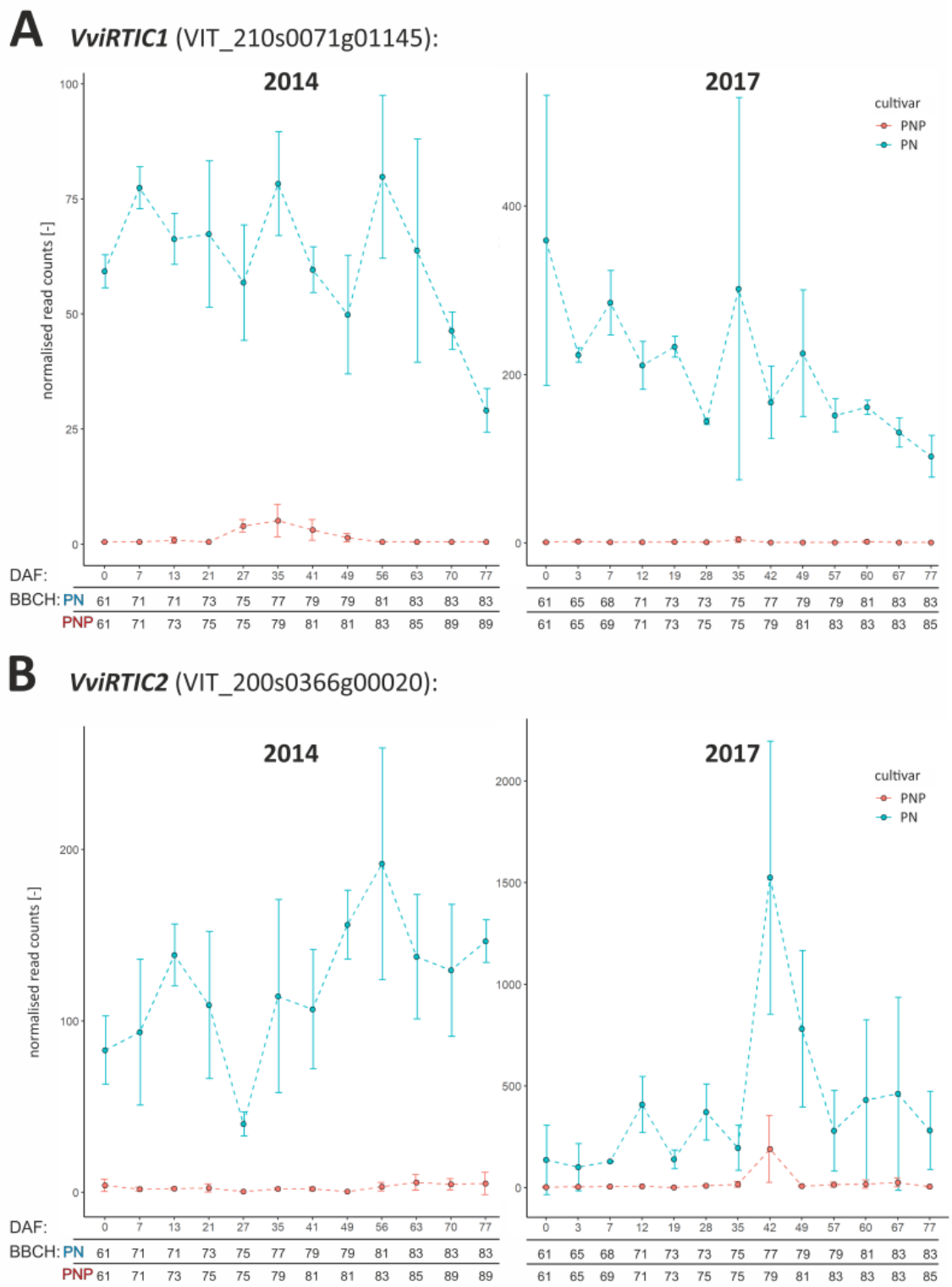
370 patterns of the 10 remaining DEGs of the putative ripening time control gene set are shown
371 in Additional file 2: Figure S3.
372 Data on the set of 12 putative ripening time controlling genes are collected in Table 3, with a
373 focus on relative up- or downregulation of expression before veraison of PNP. Also, a short
374 description of the differences of the expression patterns before, at and after veraison of PN
375 and PNP is included.
376
377 **Table 3:** Collection of features of the set of 12 genes classified as potential regulators based
378 on their differential expression before veraison of PNP. The detailed expression patterns are
379 shown in Figure 5 and Additional file 2: Figure S3. Details on annotation are listed in
380 Additional file 1: Table S13.

gene ID	gene symbol	functional annotation (transferred via BLASTp)	relative transcriptional change	
			pre veraison of PNP	before, at or after veraison of PN & PNP
VIT_205s0051g00590	*VviPL1*	pectate lyase 8	upregulated in PNP	peaks before veraison, goes down after veraison
VIT_205s0077g01980	-	uncharacterized protein LOC100248252	upregulated in PNP	peaks before veraison, stays up after veraison
VIT_206s0004g00070	*VviEXPA5*	expansin A10	upregulated in PNP	upregulated in PN, strong peak before veraison
VIT_206s0009g02560	*VviPME10*	pectinesterase 2	upregulated in PNP	off in PN, may come up later after veraison
VIT_209s0018g01490	-	methanol O-anthraniloyltransferase	upregulated in PNP	goes up before veraison, further up after veraison
VIT_210s0071g01145	*VviRTIC1*	plant protein with Domain of Unknown Function 789	upregulated in PN	off in PNP
VIT_213s0067g02930	*VviEXPA14*	expansin A8	upregulated in PNP	upregulated in PN, peak before veraison, down after
VIT_216s0013g00880	*VviOLE5*	oleosin 1	upregulated in PNP and PN	peaks before veraison, low consistency between years
VIT_216s0022g00960	*VviGRIP28*	ripening-related protein-like precursor	upregulated in PNP	goes up before veraison, stays up after veraison
VIT_216s0098g01170	*VviHDZ28*	homeobox-leucine zipper protein ATHB-12	upregulated in PN	up after flowering, goes down long before veraison
VIT_200s0366g00020	*VviRTIC2*	cysteine-rich receptor-like protein kinase 10	upregulated in PN	off in PNP
VIT_200s0956g00020	*VviLEC1*	nuclear transcription factor Y subunit B-6	upregulated in PNP and PN	peaks before/at verais., low consistency between years

381

382 The expression patterns derived from RNA-Seq confirm each other and also allow digital
383 quantification of low transcript accumulation levels. Nevertheless, three genes were chosen
384 for confirmation via qRT-PCR, namely *VviRTIC1*, *VviRTIC2* and *VviERF027* (VIT
385 216s0100g00400). *VviERF027* was included to cover a gene that displays, in different
386 samples of the time series, differential expression as well as equally high expression in PN
387 and PNP. The results obtained by qRT-PCR are fully congruent with the data from RNA-Seq
388 (see Additional file 2: Figure S4). Thus, differential gene activity in a developmental pattern
389 and in a genotype specific way has been detected by RNA-Seq as well as qRT-PCR.

390



391

392 **Figure 5:** Expression patterns of *VviRTIC1* (VIT_210s0071g01145) in (A) and *VviRTIC2*
393 (VIT_200s0366g00020) in (B) from RNA-Seq data of PN (blue) and PNP (red). Error bars
394 display the standard deviation of triplicates. Left, expression profile from 2014. Right,

395 expression profile from 2017. The y-axis represents the read counts from the output of
396 DESeq2. The x-axis represents the development stages in days after onset of flowering (DAF).
397

398 **Discussion**

399 One of the first detectable mentions of the cultivar 'Pinot Precoce' in connection with the
400 synonym '(German) Früh Burgunder Traube, translated: Early Burgundy Grape' (PNP) is in
401 the French book "Ampelographie retrospective" [37]. PNP is considered to be closely related
402 to 'Pinot noir' (PN) grapes and, here, we have confirmed the clonal relationship of PN and
403 PNP by 24 well distributed genomic SSR markers. Although this does not prove that PN is the
404 ancestor, it is very likely that PNP was derived from the cultivar PN by somatic mutation as
405 suggested earlier [36]. We used these two isogenic cultivars, that are distinguished by a clear
406 duration of berry formation phenotype, to analyze changes in gene expression throughout
407 berry development. The aim was to identify candidate genes that control the speed of berry
408 development and veraison timing. Samples from inflorescences as well as from forming and
409 ripening berries were collected from the onset of flowering until after PN and PNP veraison
410 in 2014 and 2017. These samples were subjected to RNA-Seq analyses in two well resolved
411 time series.
412

413 **Phenotypic differences between the cultivars PN and PNP**

414 The data from 4 years of careful assessment of the BBCH developmental stages of PN and
415 PNP at the same location validate earlier observations from viticulture [36] that lead to
416 establishment of PNP as a distinct grapevine cultivar in north European wine growing
417 countries. Berry formation lasts about two weeks less in PNP, is clearly accelerated
418 compared to PN and results in PNP entering veraison approximately two weeks earlier than
419 PN (Figure 1A, Table 1). It is reasonable to assume that this acceleration affects berry

420 formation throughout, from immediately after fruit set until veraison. Functionally, this
421 hypothesis implies that the genes that are responsible for the control of timing of berry
422 development, and for the establishment of the phenotypic difference between PN and PNP,
423 should be acting already very early in berry development, starting at least shortly after
424 flowering and at or even before BBCH61 to BBCH79. At the end of ripening (harvest), the
425 berries of PNP reach high sugar content earlier within the season when compared to PN.
426

427 **General validation of the RNA-Seq dataset**

428 To estimate overall data quality, the expression profiles obtained from PN and PNP were
429 correlated for the two sampled years, 2014 and 2017. Pearson correlation was moderate,
430 but this is expected considering the conditions of the free field environment. Exposure of the
431 vines to external factors like biotic or abiotic stressors, including weather conditions that
432 differ significantly between the years, also affect the transcriptome which reduces the level
433 of correlation [38]. In a PCA, almost all data points lie on the same intended track, and
434 biological replicates (subsamples) from both years are located close to each other. The main
435 actors, which predominantly influence the variance in the dataset, are genes related to cell
436 wall modification, secondary metabolism, wounding and hormone signaling. These gene
437 categories fit locical expectations since berry development is known to (i) be controlled by
438 hormones, (ii) require new cell walls, and (iii) be accompanied by accumulation of
439 secondary/specialized metabolites [24, 26]. These initial results validated the quality of the
440 dataset and indicated clearly that sampling of biologically closely related material for the
441 subsamples/triplicates was successful.
442
443

444 **Co-expression analysis shows similar gene expression clusters between cultivars and years**

445 To further validate the data with respect to comparability as well as reproducibility between
446 the two years, related gene expression profiles were identified among all genes by clustering
447 the data from the four different time series. Generally, clusters of the same genes with
448 similar expression patters over time were observed for both cultivars and both years. Also,
449 the cluster analyses for gene expression patterns among the years 2014 & 2017 in only PN
450 and in only PNP, confirmed comparability of the gene expression patterns obtained in these
451 two years. Detailed inspection revealed clusters representing expression profiles (and
452 clusters of genes) with and without an environmental influence. Especially the cultivar-
453 specific clusters C1_PN/PNP, C7_PN/PNP and C11_PN/PNP stand out. Comparison of the
454 expression profiles for PN to those of PNP in these clusters identified a similar pattern that is
455 moved to a different time in PNP. These findings are in agreement with the shifted ripening
456 time phenology of the two cultivars discussed above. In contrast, the clusters C2_PN/PNP,
457 C5_PN/PNP, C6_PN/PNP and C12_PN/PNP display more pattern similarity among the two
458 years than among the two cultivars. Thus, the genes in these four clusters may display
459 dependence on environmental factors in their expression patterns, potentially due to
460 differences in the weather conditions between the two years studied. Strong environmental
461 effects on gene expression patterns have also been described for grapevine berry
462 development at 11 different environments (vineyards) from northern Italy [39]. The
463 remaining other PN/PNP clusters C0, C3, C4, C8, C9 and C10 display highly similar expression
464 profiles ofer al four conditions. The genes included in these clusters are probably less
465 affected by environmental factors and/or the genotypic difference between PN and PNP. We
466 conclude that our RNA-Seq results and expression level comparisons between two years are
467 based on valid data.

468 When the genomic location of the DEGs is analyzed, a genome region on chromosome 16
469 comes into focus. In this region, 54 of the DEGs from the set of 1,923 intersecting DEGs
470 (Table 2) are located. Of these, 28 encode stilbene synthases [40] that are all up-regulated
471 after veraison of PNP (BBCH83). Stilbenes are a group of phenylpropanoid compounds (that
472 includes resveratrol) which are detected in many plants, which often accumulate in response
473 to biotic and abiotic stresses, and which are formed as a basic structuce by the key enzyme
474 stilbene synthase. The genome region fits to a major QTL (Ver1) for "timing of the onset of
475 veraison" on chromosome 16 [8]. It remains to be determined if this aggregation of DEGs is
476 by chance. Potentially, the observation is biased by co-regulation of a large number of
477 closely linked stilbene synthase genes.
478
479 **Differentially expressed genes throughout berry development and identification of**
480 **veraison-specific genes**
481 Differential gene expression analysis and subsequent filtering revealed 1,923 DEGs between
482 PNP and PN. DEG detection was based on a comparison of samples taken from the two
483 cultivars at very similar DAF. As expected for the characterized phenotype, PNP reaches
484 veraison when PN is still in the phase of berry formation. Consequently, the strong increase
485 in the number of detected DEGs shortly before and at veraison of PNP results from the
486 different developmental stage of PNP compared to the lagging PN. Subsequently, when also
487 PN enters veraison, the number of DEGs declines (note that Figure 4 uses a logarithmic
488 scale). A list of 388 genes that show up in both years with a veraison-specific expression
489 pattern was extracted and compared to published results. Interestingly, about 81.5% of the
490 388 PN/PNP veraison-specific genes were also described in the 4,351 differentially expressed
491 genes between the table grape cultivar '8612-CTR' (wild type) and its early ripening bud

492 mutation '8612-ERN' [27]. Also, analyses of berries from the cultivars 'Cabernet Sauvignon'
493 and 'Pinot Noir' by RNA-Seq identified a gene set of 5,404 genes marking the onset of berry
494 ripening [28]. This set covers 51.5% of the 388 PN/PNP veraison-specific genes (Additional
495 file 1: Table S14). Several "switch genes" which are supposed to encode key regulators of the
496 developmental transition at veraison [34, 41] are also included in the 388 veraison-specific
497 gene set (Additional file 1: Table S14). We conclude that the PN/PNP veraison-specific set of
498 388 genes represents a core set of genes that are relevant for executing the switch from
499 berry formation to berry ripening. As a relatively small gene set was detected that still
500 displays high overlap to those found by other studies that addressed similar biological
501 questions, indicates that the specific experimental setup and implemented filters used here
502 are appropriate to remove unrelated genes. Here, the comparison of "wildtype to mutant"
503 RNA-Seq results in isogenic background between PN and PNP, reduced environmentally
504 controlled transcriptome differences by sampling in the same vineyard/location, and dense
505 time course sampling together with high RNA-Seq read coverage allowed good resolution
506 power.
507 In order to check for potentially co-expressed genes within the veraison-specific gene set,
508 the memberships of these genes in the PN/PNP cluster analysis were investigated. A total of
509 48 veraison-specific DEGs were detected in cluster C6_PN/PNP (contains 914 genes). These
510 48 genes include several prominent ripening-related genes like *VviGRIP61*
511 (VIT_201s0011g05110), *VviMYBA8*, *VviMRIP1* (VIT_205s0049g00760, [42]), *VviGRIP4*
512 (VIT_205s0049g00520) and *VviGRIP28*. The ~20 *VviGRIP* genes were previously detected by
513 differential cDNA screening as ripening-induced genes in grape [43]. Another relevant cluster
514 is C5_PN/PNP (contains 263 genes) which includes 37 of the 388 veraison-specific DEGs.
515 Among these are *VviMYBA1*, *VviMYBA2*, *VviMYB15* and *VviGRIP22* (VIT_206s0004g02560).

516 The two clusters C5_PN/PNP and C6_PN/PNP show quite similar patterns (Additional file 2:
517 Figure S1A). It was, at first, not obvious which difference has forced CLUST to put a given
518 gene in either C5_PN/PNP or C6_PN/PNP. However, a comparison of the expression patterns
519 of *VviMYBA2* (in C5_PN/PNP) and *VviMYBA8* (in C6_PN/PNP; see Additional file 2: Figure S2
520 for a heatmap) shows that there are borderline cases regarding assignment to either
521 C5_PN/PNP or C6_PN/PNP.
522 In total, 22 genes encoding R2R3-MYB TFs were found among the 1,923 intersecting DEGs.
523 Based on the timing of expression in PN and PNP, the 22 *R2R3-MYB* genes can be classified
524 into three groups (Additional file 2: Figure S2). The first group is represented by *VviMYB24*
525 which is expressed during early flowering (BBCH61) but switched off already at the transition
526 from flowering to berry formation (BBCH71). *VviMYB24* is potentially orthologous to a group
527 of three *A. thaliana R2R3-MYB* genes (*AtMYB21/24/57*) that are expressed in flowers and
528 which function redundantly to regulate stamen development in the context of jasmonate
529 action [44]. It is tempting to speculate that *VviMYB24* has a similar function in grape.
530 The second group covers about 15 *R2R3-MYB* genes that are expressed during berry
531 formation and pre-veraison (BBCH71 to 77). This group includes *VviMYBF1* which regulates
532 flavonol biosynthesis [45], and *VviMYBPA5* as well as *VviMYBPA7* which belong to the clade
533 of *AtTT2*-related genes that control proanthocyanidin (PA, flavan-3-ol) biosynthesis [7, 46-
534 48]. The other *R2R3-MYB* genes in this group are less well characterized although there are
535 functions described for some of them, e.g. *VviMYBC2-L3* (VIT_214s0006g01620) as repressor
536 of specific branches of the phenylpropanoid pathway [49].
537 The third group of *R2R3-MYB* genes is active starting at veraison (after BBCH81) and covers
538 about six genes. Among them are the anthocyanin accumulation controlling genes,
539 *VviMYBA1*, *VviMYBA2, VviMYBA3* and *VviMYBA8* for which there is good evidence that they

540 trigger anthocyanin biosynthesis [50]. Since PN and PNP are red berry cultivars, activity of
541 the TF genes that direct anthocyanin accumulation is expected. In addition, this group
542 includes *VviMYB14* and *VviMYB15* that are supposed to regulate the stilbene biosynthetic
543 pathway [51]. With regard to the heatmaps (Additional file 2: Figure S2) and the analyses of
544 the DEGs in this study in general, it should be noted that while the resolution within the
545 developmental program and time is quite good, our data do not resolve the exact location of
546 gene expression. Therefore, it remains to be determined if the expression detected is
547 derived from berry skin, flesh, the seed or other tissues/cells.
548
549 **Putative ripening time control genes acting early in berry development**
550 To focus on genes that are contributing to the acceleration of berry formation in PNP, and/or
551 to the control of timing of veraison, we selected DEGs detected at time points prior to
552 veraison of PNP (Table 2, Figure 4). This resulted in a set of only 12 genes that are potentially
553 involved in the regulation of ripening time (Table 3). According to our hypothesis that the
554 genes relevant for acceleration of berry formation in PNP, which cause the earlier onset of
555 ripening in PNP, should be acting from at least shortly after flowering, we designated this set
556 of genes as "ripening time controlling". However, genes that encode components of the
557 respective regulatory networks and target genes of regulators including secondarily affected
558 DEGs are surely included as well [52]. The 12 putative ripening time control genes, the DEGs
559 detected before veraison of PNP, encode proteins related to auxin action, pectin processing
560 enzymes related to cell wall modification, TFs from the HD-Zip as well as NF-Y/LEC families, a
561 cysteine-rich receptor-like protein kinase, an oleosin, and proteins with domains of unknown
562 function.

563 The expression patters of this set of differentially expressed genes, and also the complete
564 dataset of DEGs detected (Table 2), was screened for genes that were higher expressed in
565 PNP than in PN. However, although there are many genes that display earlier upregulation of
566 expression in PNP than in PN in the context of earlier veraison, none of the 12 "early
567 differential" putative ripening time control genes was significantly higher expressed in PNP
568 than in PN already at or early after flowering (Table 3). Also, among the other DEGs, no gene
569 with such a differential expression pattern was detected. Such early gene activation in PNP
570 compared to PN could hint at a dominant regulator that promotes faster ripening, but the
571 data are more in favor of loss of an inhibitor of fast ripening.
572 The two genes assigned to auxin signaling by VitisNet (vv30003) encode expansins (*VviEXPA5*
573 and *VviEXPA14*, [53]). Expansins are known to be involved in fruit ripening through cell wall
574 expansion and cell enlargement [54]. Auxin can delay the onset of veraison and ripening
575 processes in grapevine [19-21]. Since reduced expression of genes from the auxin signaling
576 pathway may indicate reduced auxin action due to lower auxin levels, the accelerated entry
577 of PNP into veraison might be initiated by reduced auxin levels. Additionally, the genes
578 *VviPL1* (pectate lyase 1 [55]), *VviPME10* (pectin methylesterase 10, VIT_206s0009g02560)
579 *VviGRIP28* (encoding a pectin methylesterase inhibitor precursor-like protein) are also
580 related to cell wall processes, indicating that cell wall modification is an important target
581 process also prior to veraison [54]. The gene *VviGRIP28* was also detected within a veraison-
582 specific meta-QTL designated ver/ph16.1 [9]. It remains to be determined if this correlation
583 has a functional basis.
584 The two genes in the set of 12 that encode TFs are *VviHDZ28* (VIT_216s0098g01170, [56])
585 and *VviLEC1* (VIT_200s0956g00020, [57]). The *V. vinifera* gene VIT_216s0098g01170 that has
586 been designated *VviHDZ28* has also been considered as a homolog of *AtHB12* (At3g61890),

587 but it seems that *VviHDZ07* (VIT_202s0025g02590) and *VviHDZ27* (VIT_215s0048g02870) are
588 more similar to *AtHB12*. In these cases, which lack clearly assignable homologs, transfer of
589 functional information reaches its limits and might be restricted to concluding that *VviHDZ28*
590 is important for organ development in *Vitis*. The gene *VviLEC1* is one of three genes in *V.*
591 *vinifera* which are homologs of *AtLEC1* (At1g21970, NF-YB9) and *AtL1L* (LEC1-like,
592 At1g21970, NF-YB6). LEC1 and L1L are central regulators of embryo and endosperm
593 development. They control, among other processes, embryo morphogenesis and
594 accumulation of storage reserve [58]. It is tempting to speculate that the reason for the
595 detection of *VviLEC1* among the 12 putative ripening time control genes is that also seed
596 development needs to be accelerated in PNP compared to PN. This would explain earlier and
597 higher expression of *VviLEC1* in PNP compared to PN as observed (Additional file 2: Figure
598 S3G). Consequently, *VviOLE5* (VIT_216s0013g00880, encoding an oleosin involved in oil body
599 formation [59]) would fit into the picture as relevant for lipid storage during seed
600 development. According to the proposed enzyme function as an alcohol acyltransferase by
601 the protein encoded by VIT_209s0018g01490 involved in volatile ester formation [60], this
602 gene could play a similar role. For the gene VIT_205s0077g01980 no functional annotation is
603 available (uncharacterized protein), although homologs exist throughout the Magnoliophyta.
604

605 **Candidates for causal genes explaining the difference between PN and PNP**

606 Among the 12 putative ripening time control genes, of which 10 are discussed above, two
607 are especially interesting. Detailed analyses of the full set of DEGs, visualized in Figure 4,
608 resulted in the identification of *VviRTIC1* and *VviRTIC2*, that could possibly be centrally
609 involved in the accelerated berry development and earlier beginning of ripening in PNP
610 compared to PN. The special feature of the expression patters of the two genes (Figure 5,

611 Table 3) is that both are differentially expressed already at the first time point analyzed
612 which was selected to hit the BBCH stage 61 (flowering before full bloom, DAF zero (0)).
613 Also, both genes are only barely expressed in PNP in both years studied, while expression in
614 PN is high at almost all time points. *VviRTIC1* is annotated to encode a protein containing a
615 domain of unknown function (DUF789), while *VviRTIC2* is annotated to encode a "cysteine-
616 rich receptor-like protein kinase". The best BLASTp hit to *A. thaliana* protein sequences
617 indicates that it is related to At4g23180/AtCRK10, but a closer inspection shows that
618 similarity to At4g05200/AtCRK25, At4g23160/AtCRK8 and At4g23140/AtCRK6 is almost as
619 high. This ambiguity, and also the fact that the *V. vinifera* genome contains several genes
620 related to *VviRTIC2* (e.g. VIT_210s0071g01200, VIT_202s0087g01020 or
621 VIT_203s0017g01550 as listed by PhyloGenes), complicates transfer of functional
622 information. CRKs are a subgroup of plant receptor-like kinases [61] and are encoded by a
623 family of 44 genes in *A. thaliana*. In a systematic analysis of the functions of *A. thaliana* CRKs,
624 evidence was collected for involvement in the control of plant development, biotic and
625 abiotic stress responses, photosynthesis as well as stomatal regulation [62]. This systematic
626 phenotypic screen of a large set of T-DNA insertion mutants revealed distinct phenotypes for
627 various of the CRK genes, but assignment of a molecular function to individual CRKs beyond
628 recognition of unknown ligands and signal transmission by phosphorylation remains a large
629 challenge.
630 As pointed out above, it is very possible that the genes we have identified are part of a
631 genetic pathway that controls timing of berry development in *V. vinifera*, but that we have
632 hit genes in a downstream part of this pathway. The relevance of the two candidate genes in
633 the causal genetic difference between PN and PNP remains to be determined. Phase-
634 separated genome sequences of the cultivars will be required to resolve the genome

635 structure of both alleles of *VviRTIC1 and 2* the genes in PN and PNP for an informative
636 comparison. In future studies, we will address this question, for example by long read DNA
637 sequencing.
638
639 **Conclusions**
640 This study detected 1,923 DEGs between the Pinot cultivars PN and PNP. The two clonal
641 cultivars display a phenotypic difference in berry development timing where PNP reaches
642 from full bloom to veraison faster than PN. We defined 388 DEGs as veraison-specific and 12
643 DEGs as putatively controlling ripening time. The relatively small number of veraison-specific
644 genes displays a very high overlap with results published for similar studies (see Additional
645 file 1: Table S14) and could be used for studying a phytohormone network that is acting
646 similarly in PN and PNP, but accelerated in PNP. Additionally, the ripening time control genes
647 identified here might offer access to a set of genes putatively important for triggering or
648 delaying the start of berry ripening in grapevine. Further investigations are needed to
649 elucidate structural differences in the genomes, the function of the observed DEGs, and their
650 role in shifting the onset of ripening in grapevine.
651
652 **Material and Methods**
653 **Plant material and analysis of clonal relation**
654 The grapevine (*Vitis vinifera* subsp. *vinifera* L.) cultivar PNP (Pinot Precoce Noir, VIVC No.
655 9280) is early ripening and has been described to be related to the cultivar PN (Pinot Noir,
656 VIVC No. 9279) [35] that ripens later than PNP. To prove the clonal relation, DNA from both
657 cultivars was genotyped utilizing 24 polymorphic SSR markers (VVS2, VVMD7, VVMD5,
658 VVMD32, VVMD28, VVMD27, VVMD25, VVMD24, VVMD21, VVIV67, VVIV37, VVIQ52,

659 VVIP60, VVIP31, VVIN73, VVIN16, VVIH54, VVIB01, VrZAG83, VrZAG79, VRZAG67, VrZAG62,
660 VMC4F3.1, VMC1B11) as described [63]. The two cultivars used have been identified as
661 accession DEU098_VIVC9280_Pinot_Precoce_Noir_DEU098-2008-076 and
662 DEU098_VIVC9279_Pinot_Noir_DEU098-2008-075, respectively. The tissue used for harvest
663 is indicated below and in Figure 1. Both cultivars do not belong to an endangered species
664 and were obtained and are grown in accordance with German legislation.
665
666 **Phenotypical characterization and sampling of plant material**
667 Plant material was harvested from PN and PNP grapevines trained in trellis. The plants are
668 growing at the vineyards of JKI Geilweilerhof located at Siebeldingen, Germany (N
669 49°21.747, E 8°04.678). The grapevine plants were planted with an interrow distance of 2.0
670 m and spacing of 1.0 m in north-south direction. Inflorescences, developing and ripening
671 berry samples of PNP and PN for RNA extraction were collected in two years with three
672 independent biological replicates (subsamples) each. Sampling took place at systematic time
673 points (12 time points in 2014, 13 time points in 2017), and at approx. 8 a.m. each day. In
674 2014, harvesting took place regularly every 7 days with only two exceptions (one day
675 deviation, DAF 13 and DAF 27). In 2017, harvesting was adapted to BBCH stages (Figure 1A).
676 The timeline in both years is described as days after onset of flowering (DAF), with onset of
677 flowering defined as the day at which 10% of the individual flowers have lost their caps
678 (BBCH61 [3]). For each subsample within the triplicates, material from two neighboring
679 grapevines was selected. Grapevine plants were weekly phenotyped according to BBCH
680 stage [3, 4]. Phenotyping was performed repeatedly to ensure sampling from vines of the
681 same development stage (e.g. percentage of open flowers during flowering, or berry
682 development stage) to reach uniform subsamples. The phenotypical observations were

683 summarized in Additional file 1: Table S2 and S3. From these, the durations of flowering,
684 berry formation and berry ripening as well as the resulting shifts between the cultivars were
685 calculated (Table 1). Furthermore, images from berry developmental stages of both cultivars
686 were taken in 2014 for 35, 41, 49 and 56 DAF. The sampled material was directly frozen in
687 liquid nitrogen and stored at -70°C until RNA extraction.
688
689 **RNA extraction and cDNA library construction**
690 Biological replicates, i.e. the subsamples, were ground separately under liquid nitrogen.
691 Total RNA was extracted using an RNA Isolation Kit (Sigma-Aldrich Spectrum™ Plant Total
692 RNA) according to suppliers' instructions. Quality control, determination of RIN numbers [64]
693 and estimation of the concentrations of all RNA samples was done on a Bioanalyzer 2100
694 (Agilent) using RNA Nano 6000 Chips. For RNA-Seq, 500 ng total RNA per subsample were
695 used to prepare sequencing libraries according to the Illumina TruSeq RNA Sample
696 Preparation v2 Guide. For subsamples from 2014 and 2017, 72 and 78 libraries were
697 constructed and sequenced, respectively. Enrichment of poly-A containing mRNA was
698 performed twice, using poly-T oligos attached to magnetic beads included in the Illumina kit.
699 During the second elution of the poly-A+ RNA, the RNA was fragmented and primed for
700 cDNA synthesis. After cDNA synthesis, the fragments were end-repaired and A-tailing was
701 performed. Multiple indexing adapters were ligated to the ends of the cDNA fragments and
702 the adapter ligated fragments were enriched by 10 cycles of PCR. After quality check using
703 Bioanalyzer 2100 HS-Chips (Agilent) and exact quantification by Quant-iT PicoGreen dsDNA
704 assay on a FLUOstar Optima Plate Reader (BMG LABTECH), the libraries were pooled
705 equimolarly.
706

707 **RNA-Seq**

708 Single end (SE) sequencing of the pooled barcoded libraries from 2014 was performed on an
709 Illumina HiSeq1500 in High Output mode generating 100 nt reads. For samples from 2017,
710 sequencing was done using an Illumina NextSeq500 generating 83 nt SE reads; two runs
711 were performed with the same pool of barcoded libraries from 2017.

712

713 **Processing of RNA-Seq read data**

714 Raw reads were trimmed with Trimmomatic (version 0.36) [65]. For raw reads from the year
715 2014, the following settings were used: LEADING:10 TRAILING:10 SLIDINGWINDOW:4:15
716 MINLEN:50. In addition, a collection of all available Illumina adapter sequences was supplied
717 to remove matches within the parameter 2:30:10. For raw reads from the year 2017,
718 trimming settings were set to LEADING:6 TRAILING:6 SLIDING WINDOW:4:15 MINLEN:36. All
719 trimmed reads were quality-checked via FastQC (version 0.11.8) [66]. Thus, possible adapter
720 sequences and low-quality bases were removed. All trimmed reads passing QC were mapped
721 to the reference genome sequence PN40024 (version 12Xv2) [67] using the graph-based
722 alignment tool HISAT2 (version 2.1.0) [68, 69] with no additional soft clipping. Afterwards, all
723 tagged genes (structural gene annotation: CRIBI v2.1) were counted as raw read counts with
724 FeatureCounts (Bioconductor package Rsubread version 3.8 [70]). To estimate transcript
725 abundance as a measure for gene expression, counts for Transcripts Per Kilobase Million
726 (TPM, [71]) were determined.

727

728 **Basic gene expression analyses**

729 TPM counts from the various samples were used for manual gene expression inspection, for
730 determination of the number of expressed and not expressed genes, and to calculate the

731 correlation between gene expression values from both years. Genes with a TPM value > 0
732 added up over all samples from one year were classified as expressed, conversely genes with
733 a TPM value = 0 added up over all samples as not expressed. A custom python script was
734 applied utilizing the function pearsonr from SciPy python package (v. 1.2.3) [72], which
735 calculates the Pearson correlation coefficient and the p-value for all year-to-year
736 comparisons. Expression data pairs for TPM counts per gene from both sampled years,
737 averaged over the three subsamples of each sample, were used. To test correlations and
738 relationships between expression values from the two years, where samples were harvested
739 with slightly different sampling patterns (see Figure 1A), eight equivalent time points with
740 the same BBCH stages between the cultivars of each year were selected (see Additional
741 file 1: Table S4).
742
743 **Principal component analysis**
744 To explore data similarity, a Principal Component Analysis (PCA) was calculated over all gene
745 expression values from both years and cultivars for all subsamples. All data points were
746 normalized using variance stabilizing transformation function 'vst' from the R package
747 DESeq2 (v. 1.12.4) [73]. Subsequently, the principal components were generated using
748 'prcomp' from the R package 'stats' (v. 3.5.2) [74]. The resulting PCA object, displaying the
749 main components PC1 and PC2, was plotted and exported. Additionally, genes with the
750 highest variance contribution to PC1 and PC2 were extracted separately.
751
752 **Functional annotation of genes**
753 Transfer of annotation information from other plant species, mainly *A. thaliana,* was
754 calculated using MapMan's sequence annotation tool Mercator (v. 3.6) [75, 76]. Additionally,

755 all open reading frame (CDS from *V. vinifera*/grapevine genes) sequences were aligned to
756 the non-redundant protein sequence data base RefSeq [77] with the basic local alignment
757 tool for proteins BLASTp [78] (e-value ≤ 0.001). Short descriptions of gene functions were
758 extracted and added to the gene lists in Additional file 1: Table S5, S6, S13, S14.
759 GO term enrichment for biological processes was calculated via the R package 'topGO' (v.
760 2.38.1) [79]. Subsequently, statistical reliability was calculated using Fishers exact test. All
761 Gene IDs and their corresponding GO terms were extracted from the CRIBI database
762 (http://genomes.cribi.unipd.it/DATA/V2/annotation/bl2go.annot_with_GO_description.txt).
763 All results of the GO term enrichment are deposited in Additional file 1: Table S10-12.
764
765 **Cluster analysis**
766 To reveal co-expressed genes over all four datasets, the tool CLUST (v. 1.10.8) was used with
767 default parameters [80]. As input, raw read counts from eight time points were used. These
768 time points were selected to cover the same BBCH stages of PN and PNP from the years
769 2014 and 2017 (Additional file 1: Table S4). First, all data were pre-processed as described in
770 the CLUST manual. Values from corresponding subsamples (triplicates) were combined and
771 averaged. To filter out uninformative (very low) gene expression values, an additional filter
772 was applied: genes not reaching a sample expression value > 1 in at least three conditions
773 and in at least one cultivar from one year were discarded (-fil-v 1 -fil-c 3 -fil-d 1). Afterwards,
774 the data were quantile normalized according to the RNA-Seq defaults of CLUST. Genes
775 showing a flat expression profile were filtered out by applying the default settings [80].
776
777
778

779 **Differential gene expression analyses**

780 For analyses of differentially expressed genes, DESeq2 (v. 1.12.4; R Bioconductor) was
781 employed. To test if gene expression differs significantly between two samples, the
782 likelihood ratio test nbinomLRT, included in the DESeq2 package, was used. As input, raw
783 read counts from all time points were used. Normalization factors and dispersion estimates
784 were used as described [73]. The output table contained all differentially expressed genes
785 (DEGs) and the corresponding values for baseMean, log2FoldChange (LFC), lfcSE (LFC
786 standard error), stat (difference in deviation between the reduced model and the full
787 model), p-value and PADJ (adjusted p-value). To focus on significantly differentially
788 expressed genes from the DESeq2 analyses, cut-off filters PADJ ≤ 0.05 and LFC > 2 were
789 applied.

790

791 **Confirmation of differential gene expression by qRT-PCR**

792 To verify the RNA-Seq results, four time points from 2017 were selected (DAF 0, DAF 28,
793 DAF57 and DAF77) for qRT-PCR. Synthesis of cDNA from the RNA subsamples was carried
794 out with First Strand cDNA Synthesis Kit (ProtoScript® II; NEB) according to the
795 manufacturer's instructions. The qRT-PCR assay was performed using Luna Universal qPCR
796 Master Mix (NEB) with a total volume of 20 µl. Sequences of the primers used are listed in
797 Additional file 1: Table S23. Reaction products/amplicons were detected based on SYBR
798 green via a CFX96 Real Time PCR Detection System (Bio-Rad). For each time point, three
799 biological and three technical replicates (i.e., each subsample in triplicate) were measured.
800 Cycling conditions after initial denaturation 2 min at 95°C: denaturation 5 sec at 95°C,
801 annealing/extension 30 sec at 60°C, cycled 35 times. For QC, each reaction was controlled by
802 product melt analysis (65°C - 95°C). As negative controls, no template control (NTC) and no

803 reverse transcriptase control (-RT) were measured as well using three technical replicates.
804 The polyubiquitin gene *VviUbiquitin1* (VIT_216s0098g01190) was used for normalization
805 [46]. Measurements were analyzed using the CFX Maestro V.4.1.2433.1219 (Bio-Rad) by
806 normalization via the relative quantitative ΔΔCt method.
807

808 **Selection of gene sets potentially relevant for ripening time and comparison with**
809 **literature data**

810 In order to identify gene sets from the DEGs relevant for control and implementation of
811 ripening, an intersection between the DEGs detected at all time points between both years
812 was built. To determine a subset of putatively ripening time control genes, the intersection
813 between both years covering the development stages BBCH61 (onset of flowering) to
814 BBCH79 (one developmental BBCH stage before veraison) was used (time points 2014:
815 DAF 0-35; 2017: DAF 0-42). Furthermore, a set of veraison specific genes was defined from
816 the DEGs detected at the intersection of development stages BBCH79 (one developmental
817 BBCH stage before veraison) to BBCH81 (onset of ripening / veraison; time points 2014:
818 DAF35-41; 2017: DAF42-49). To test for biological relevance of the subsets, all DEGs were
819 screened to their occurrence in similar relevant studies [9, 27, 28, 30, 34, 41, 81, 82].
820

821 **Visualization of gene numbers newly appearing as differentially expressed**

822 To visualize appearance of DEGs over time, a stacked bar plot script was set up using the R
823 package 'plotly' (v. 4.9.2.1) [83]. Each bar represents the amount of DEGs of a given time
824 point or condition. In order to track groups of DEGs newly appearing at a given time point
825 throughout the following time points, the colour shade representing the group of DEGs
826 remains the same.

827 **Pathway enrichment analysis**

828 To search for possible targets in known pathways of grapevine, a pathway enrichment
829 analysis using the tool VitisPathways [84] was performed. To achieve a reliable enrichment,
830 1000 permutations, a Fisher's exact test of $p < 0.05$ and permuted p-value < 0.1 were set.
831 Thus, all significant enriched pathway genes and their relations can be displayed in VitisNet
832 [85], a specific molecular network for grapevine.

833

834 **Heatmaps**

835 As an extension to assignment of genes to biosynthesis pathways, the genes were also
836 filtered for annotation as coding for transcription factors (TFs). This filter was based on the
837 annotation information transferred from Mercator and RefSeq (see above). To look at the
838 entire family of *R2R3-MYB* TF genes, the list of *MYB* genes identified via MapMan was
839 extended by additional grapevine *R2R3-MYB* gene family members that have been
840 characterized [6, 7]. The *R2R3-MYB* genes detected among the intersecting DEGs were
841 displayed in heatmaps addressing the four individual time series (2 cultivars, 2 years) using
842 the R package 'pheatmap' (v. 2.1.3) [86]. Predictions for phylogenetic relationships were
843 deduced from PhyloGenes v. 2.2 [87].

844

845 **Abbreviations**

846 ABA abscisic acid

847 DAF days after onset of flowering

848 DEGs differentially expressed genes

849 PNP Pinot Noir Precoce

850 PN Pinot Noir

LFC	log2fold change
PADJ	adjusted p-value
PCA	principle component analysis
TF	transcription factor
TPM	transcripts per kilobase million

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and material

The FASTQ files containing all RNA-Seq reads (PN2014, PNP2014, PN2017 and PNP2017) have been deposited at the European Nucleotide Archive (ENA) according to the INTEGRAPE guidelines under the accession numbers PRJEB39262, PRJEB39261, PRJEB39264 and PRJEB39263, respectively (see Additional file 1: Tables S19 to S22). All scripts developed for this study are available on GitHub [https://github.com/bpucker; https://github.com/jenthein].

Competing interests

The authors declare that they have no competing interests

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877

878 **Authors' contributions**

879 Sampling and phenotyping were done at the JKI (Julius Kühn-Institute, Institute for
880 Grapevine Breeding Geilweilerhof, Siebeldingen, Germany). Sequencing and data analysis
881 were performed at Bielefeld University, Faculty of Biology & Center for Biotechnology
882 (CeBiTec). Organization, supervision at Bielefeld University and parts of sampling were done
883 by DH. KH, AK and FS coordinated weekly sampling, phenotyping, and image capture for
884 documentation. Logistic work was done by KH. The design of the experiments was set up by
885 FS, DH and KH. RNA isolation and cDNA synthesis were carried out by DH and PV. PV
886 accomplished library preparation and sequencing. Quantitative RT-PCR was performed by JT
887 and DH. RT and KH supervised the work at JKI Geilweilerhof. LH performed the SSR analysis.
888 BW supervised the work at Bielefeld University. FS, KH, RT and BW acquired project funding
889 and wrote the project proposal. All bioinformatic data analyses, creation of figures, tables
890 and writing of the manuscript were performed by JT with the help of DH. JT and BW drafted
891 the manuscript. All authors have read and approved the final manuscript.

892

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Supplementary material

Additional file 1: Table S1: Clonal relation of PN and PNP confirmed by 24 SSR markers

Additional file 1: Table S2 - Sampling and phenotypical observations (BBCH) for 2014

Additional file 1: Table S3 - Sampling and phenotypical observations (BBCH) for 2017

Additional file 1: Table S4 - Pearsonr correlations of gene expression values over time between both years

Additional file 1: Table S5 - Top 500 genes influencing principal component PC1

Additional file 1: Table S6 - Top 500 genes influencing principal component PC2

Additional file 1: Table S7 - Cluster memberships of clusters obtained from the clusteranalysis PN/PNP

Additional file 1: Table S8 - Cluster memberships of clusters obtained from the cluster analysis PN

Additional file 1: Table S9 - Cluster memberships of clusters obtained from the cluster analysis PNP

Additional file 1: Table S10 - GO term enrichment for the clusters from the cluster analysis of PN/PNP

Additional file 1: Table S11 - GO term enrichment for the clusters from the cluster analysis of PN

Additional file 1: Table S12 - GO term enrichment for the clusters from the cluster analysis of PNP

927 **Additional file 1: Table S13** - Differentially expressed genes between PN and PNP in both
928 years and filtering steps
929 **Additional file 1: Table S14** - Overlap of 12 putatively ripening time control DEGs (time
930 points: DAF 0-35 from 2014 intersecting with DAF 0-42 from 2017) and 388 veraison-specific
931 DEGs (time points: DAF 35+41 from 2014 intersecting with DAF 42+49 from 2017) with
932 previous transcriptomic grapevine studies.
933 **Additional file 1: Table S15** - VitisNet enrichment for intersecting DEGs
934 **Additional file 1: Table S16** - VitisNet enrichment for regulatory DEGs
935 **Additional file 1: Table S17** - VitisNet enrichment for veraison specific DEGs
936 **Additional file 1: Table S18** - Up- & Down-regulated TFs among the DEGs
937 **Additional file 1: Table S19** - ENA sample identifier and metadata linked to the study for PNP
938 RNA-Seq reads from 2014 (PRJEB39262)
939 **Additional file 1: Table S20** - ENA sample identifier and metadata linked to the study for PN
940 RNA-Seq reads from 2014 (PRJEB39261)
941 **Additional file 1: Table S21** - ENA sample identifier and metadata linked to the study for PNP
942 RNA-Seq reads from 2017 (PRJEB39264)
943 **Additional file 1: Table S22** - ENA sample identifier and metadata linked to the study for PN
944 RNA-Seq reads from 2017 (PRJEB39263)
945 **Additional file 1: Table S23** - Sequences of primers used for qRT-PCR
946
947 **Additional file 2: Figure S1** - Cluster analyses of PN/PNP in both years (A), PN in both years
948 (B) and PNP in both years (C)
949 **Additional file 2: Figure S2** - Heatmap of all MYB-TFs gene expression among the DEGs for
950 PN and PNP in both years
951 **Additional file 2: Figure S3** - Expression patterns of genes from the ripening-regulatory gene
952 set
953 **Additional file 2: Figure S4** - Confirmation of expression data from RNA-Seq by qRT-PCR
954
955

956 **References**

957

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