Loss Of Diel Circadian Clock Gene Cycling Is A Part Of Grape Berry Ripening

Running head: Loss of circadian clock cycling in ripening grapes

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Loss Of Diel Circadian Clock Gene Cycling Is A Part Of Grape Berry Ripening

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Abstract

Diel cycles of gene expression are thought to adapt plants to 24 h changes in environmental conditions. The circadian clock contributes to this process, but less is known about circadian programs in developing reproductive organs. Whilst model plants and controlled conditions have contributed greatly to our knowledge of circadian clock function, there is a need to better understand its role in crop plants under field conditions with fluctuating light and temperature. Here, we investigated changes in the circadian clock during the development of grape berries of *Vitis vinifera* L. We found that the transcripts of circadian clock homologs had high amplitude oscillations prior to, but not during, ripening. As ripening progressed, the amplitude and rhythmicity of the diel oscillations decreased until most transcripts tested had no significant fluctuation over the 24 h cycle. Despite this loss of rhythmicity, the majority of circadian clock genes investigated were expressed at or near their abundance at the nadir of their pre-ripening oscillation, although the berries remained transcriptionally active. From this, it can be concluded that cycling of the canonical circadian clock appears unnecessary for berry ripening. Our data suggest that changes in circadian clock dynamics during reproductive organ development may have important functional consequences.

Key words: circadian rhythms, fruit development, viticulture.

Introduction

Plants are exposed to regular fluctuations in environmental conditions caused by the 24 h cycles of day and night. The 24 h cycles of day and night have selected for the evolution of circadian clocks, which contribute to the fitness of plants (Dodd et al. 2005). Circadian clocks are biological timing devices that produce a cellular measure of the time of day. This is used to align the phase of cellular processes with the external time, which is thought to confer advantages by scheduling metabolic and developmental processes according to the environmental conditions. Fruits and their products are of nutritional, economic and societal importance. As the circadian clock has a large influence on environmental responses and plant productivity (Dodd et al. 2005, Hotta et al. 2007, Graf et al. 2010), a better understanding of its function during fruit development is of interest both scientifically and commercially.

Circadian rhythms are self-sustaining biological cycles that have a period of approximately 24 h. In *Arabidopsis thaliana* (Arabidopsis), the circadian clock is entrained to 24 h fluctuations in light conditions, temperature and metabolic state (Haydon et al. 2013, Bläsing et al. 2005, Michael et al. 2003, Somers et al. 1998). Much of our knowledge of plant circadian clock architecture derives from the model organism Arabidopsis, where the circadian oscillator is thought to comprise several interlocking transcription-translation feedback loops predominated by negative feedback steps. This network includes a core feedback loop that interacts with morning and evening phased loops (see Pruneda-Paz and Kay 2010 for a detailed description). Many components of the circadian oscillator are transcription factors, which bind to sets of gene promoters to produce genome-wide circadian rhythms of transcript abundance.

In Arabidopsis, approximately 30-40% of the transcriptome is circadian-regulated (Michael and McClung 2003, Covington et al. 2008), and about 90% of transcripts can have a 24 h oscillation when a range of different environmental cycle conditions are considered (Michael et al. 2008). Therefore, circadian and diel regulation has an incredibly pervasive influence upon gene expression in plants. The circadian clock contributes to a broad range of processes including growth, the scheduling of flowering, photosynthesis, primary carbohydrate metabolism, and responses to various abiotic and biotic cues (Creux and Harmer 2019, Doherty and Kay 2010). Together, this is thought to align metabolism and development with diel environmental fluctuations which, in turn, increases plant performance and fitness.

A distinction between the circadian organization of plants and animals is that in plants, each cell appears to have a semi-autonomous circadian oscillator (Paajanen et al. 2021). Whilst there is weak intercellular coupling of circadian rhythms in leaves and a dominance of the apex and vasculature upon rhythms elsewhere (Endo et al. 2014, Takahashi et al. 2015), the level of coordination between tissues and organs varies considerably, which results in tissue and organ-specific levels of circadian organization (Sorkin and Nusinow 2021, Uemoto et al. 2023). Therefore, the circadian system can have organ- and tissue-specific specializations. For example, in Arabidopsis roots the operation of the circadian oscillator is different because only the morning-phased loop

seems to operate, driving the expression of a smaller number of clock output genes compared to shoots (James et al. 2008). In this context, an intriguing knowledge gap is that we know less about changes in the dynamics of the circadian oscillator during the development of reproductive organs. In sunflowers, the circadian clock regulates the flower head orientation (Atamian et al. 2016) and flower development (Marshall et al. 2022), with the circadian regulation of flower head orientation contributing to pollinator success (Creux et al. 2021). Furthermore, the circadian clock regulates floral scent emission and consequently floral attraction (Fenske et al. 2015, 2018). Therefore, circadian regulation of reproductive organs influences processes that confer reproductive fitness.

Grape berries represent an excellent model within which to investigate circadian clock dynamics in developing fruit, due to their well-characterized developmental stages and quality of genomic resources. Furthermore, the process of grape berry development is of considerable economic importance and understanding its characteristics opens opportunities for improving agronomic and compositional characters, and for adapting to climate change. Grape berry development consists of three phases (Ollat et al. 2002). The first phase following fruit set is characterised by cell division followed by cell expansion. During this phase a range of metabolites accumulate that are important to winemaking, such as malic and tartaric acids, and tannins. Following this is a lag phase where berry size does not substantially increase, after which a second phase of berry expansion occurs. The berries roughly double in size during ripening through cell expansion, soften, and rapidly accumulate high concentrations of the hexoses glucose and fructose (in roughly equal amounts), and accumulate skin colour pigments (anthocyanins) in red grapes.

Two previous studies suggested that diel (24 h) cycles of gene expression dynamics change during the course of grape berry development (Rienth et al. 2014, Carbonell-Bejerano et al. 2014), but full time-series experiments across multiple developmental stages are necessary to understand these observations. Furthermore, detailed developmental studies have reported changes in circadian clock gene expression during vine development and grape berry ripening (for example Fasoli et al. 2012, Ghan et al. 2017, Fasoli et al. 2018, Tornielli et al. 2023), but these rely on a single timepoint within each sampling day so the data cannot be interpreted to understand circadian clock dynamics. This motivated us to conduct a detailed investigation of the nature of circadian clock component dynamics in field-grown grapes, under naturally fluctuating conditions. We wished to determine the relationship between circadian clock dynamics and berry developmental stage and compare this in red- and white-skinned grape cultivars. We selected field conditions and vineyard-grown grapes as the model for our experiments because this provides the most representative information about circadian clock dynamics during commercial cultivation. We identified extensive changes in circadian clock dynamics at the point of veraison (used here as the term denoting the commencement of ripening) and interpret these data in the context of abiotic stress conditions and sugar accumulation.

Results

The diel patterns of accumulation of nine transcripts encoding putative circadian clock and clock output components were studied in grape berries and leaves (*Vitis vinifera* L.) at specific stages of development. Whilst days post-flowering provides a useful measure of developmental stage throughout berry development, the concentration of total soluble solids (TSS) measured as degrees Brix by refractometry is a more useful measure during the ripening phase. Berry TSS concentration changes throughout the ripening phase and increases rapidly after veraison, reflecting the large increase in hexose content (Coombe 1992). As Brix values post-veraison predominantly indicate changes in the concentration of the major metabolites in ripening berries, it is a useful measure of ripening stage.

Circadian clock transcripts have greatly reduced amplitude after veraison

First, we investigated changes in the diel dynamics of transcripts encoding circadian clock components at six different development stages in the Shiraz cultivar berry (Fig. 1A, B; Table 1, Supplemental Table 1). The identities of circadian clock and associated genes, and other marker genes, was determined by sequence similarity (Supplemental Table 2). Splice variants are possible for seven of the 12 genes, but the primers used should detect 34 out of the 36 possible variants and thus provide an accurate assessment of total transcript abundance (Supplemental Table 3). For the first three sampling times, the Brix remained below 6^{o}. By the PostV-I sample, the Brix increased to 16.17^{o} indicating a rapid increase in hexose concentration (Table 1). By the time of the final sampling, PostV-III, the Brix had increased to 23.35^{o}. Up to and including the veraison sample, the amplitude of oscillation of clock components over the diel cycle remained pronounced and similar at all timepoints, for all the transcripts except for *VviLIP1* (Fig. 1C, Fig. 2). Here, we define amplitude as the maximum displacement of the oscillation from its mean level (Paajanen et al. 2021). For *VviLIP1*, the amplitude of cycling decreased considerably after the first PreV-I timepoint (Fig. 1C, Fig. 2). In contrast, the amplitude of the oscillation for *PRR9* increased during the period up to, and including, veraison (Fig. 1C, Fig. 2).

After veraison the patterns of accumulation of all transcripts changed such that by the time of the last sampling (PostV-III), three of the nine transcripts had no statistically significant change over the 24 h period (Fig. 1C, Supplemental Table 4). For those transcripts that did oscillate, the amplitude was lower and the phase was not aligned with the pre-veraison pattern. Notably, the pre-veraison cycling pattern of *VviPRR7a* and *VviRVE1* persisted for longer than for the other transcripts, even though there was a similar decrease in amplitude. The decrease in amplitude of the oscillations during development, and particularly after veraison, is quite stark (Fig. 2). The post-veraison period is characterised by changes in a range of physical and biochemical parameters (Coombe, 1992), including a rapid increase in total soluble solids concentration as shown by increasing Brix levels (Table 1). This rapid increase in hexose concentration appeared to coincide with the decrease in amplitude of oscillation of transcripts encoding circadian clock components. Interestingly, *VviPRR7a* cycled until the final, PostV-III sampling period, but the time of its peak transcript accumulation changed after veraison. Before veraison the abundance peaked at 1pm, but after veraison (PostV-I and PostV-II

samples) the peak occurred at 9pm. A similar change occurred in the timing of *VviPHYC* accumulation, although the expression level post-veraison was much lower (Fig. 1C).

For most of the circadian clock-associated transcripts, their abundance during the post-veraison period was lower than the pre-veraison maximum. For example, at the PostV-III stage the level of *VviPRR7a*, *VviGI*, *VviLIP1* and *VviPHYC* transcripts was consistent with their lowest abundance during the pre-veraison period (Fig. 1C). The expression of *VviLHY*, *VviELF4*, and *VviRVE1* post-veraison was reduced so that it was positioned between the pre-veraison minimal and maximal expression levels. In contrast, *VviFKF1* was expressed consistently at the PostV-III stage, at a level similar to the top of its range of diel fluctuation during the pre-veraison period (Fig. 1C).

Berry transcriptional machinery remains functional throughout ripening

As there was a decrease in the abundance and amplitude of oscillation of the nine circadian clock-related transcripts during the post-veraison period, we wished to determine whether the grape berry transcription machinery was still functional during this period. Therefore, we examined the abundance of transcripts that are thought to be unrelated to the circadian clock, and instead related to berry ripening. These three transcripts (*VviTL1*, *VviCSLG*2 and *VviIAA19*) increased in abundance after veraison and did not appear to have diel oscillations, suggesting that controlled transcription continued to occur in the expected manner after veraison (Fig. 3). Therefore, the reduced amplitude of circadian clock transcript cycling is not due to a general shutdown in transcription during berry ripening.

Post-veraison attenuation of circadian clock gene expression is a general phenomenon in grape berries

We wished to determine whether the changes in circadian clock dynamics during berry ripening are conserved across grape varieties. To test this, we studied circadian clock transcript cycling in the white-skinned cultivar Fiano. This involved monitoring of transcript accumulation over a 24 h period, as for Shiraz, but at only two developmental stages (pre and post-veraison; Table 1, Supplemental Table 1). Based on the time after flowering and ^{o}Brix, these two developmental stages for Fiano relate most closely to the Shiraz PreV-II and PostV-III stages. For most transcripts, the range of copy numbers was similar in Shiraz and Fiano. We identified in Fiano a similar change during ripening in the amplitude of oscillation of circadian clock-associated transcripts, with a few exceptions (Fig. 4). Post-veraison, only one of nine transcripts, *VviRVE1*, had statistically significant differences in accumulation across the 24 h period and maintained a reasonably large amplitude (Fig. 5). This was generally similar to our experiment with Shiraz. As for Shiraz at the PostV-III timepoint, *VviPRR9* in Fiano was expressed at a high and constant level post-veraison (Fig. 4). In contrast, *VviFKF1* was expressed at a consistently high level in Shiraz PostV-III (Fig. 1C) but at a low to mid-range level in Fiano post-veraison (Fig. 4).

As with Shiraz, the upregulation of three ripening-associated transcripts (*VviTL1*, *VviCSLG2* and *VviIAA19*) suggests that the transcriptional machinery remains functional in Fiano berries post-veraison (Supplemental Fig. 3). This reinforces the interpretation that the change in the diel cycling of circadian clock transcripts was due to a specific change in circadian clock dynamics, and not due to a general loss of mRNA synthesis or catabolism during ripening.

Post-veraison circadian clock disruption is grape berry-specific

To determine whether the disruption to the circadian clock transcript rhythm in post-veraison berries was an organ or plant-wide phenomenon, the accumulation of three circadian clock-associated transcripts was examined over a diel cycle in mature Shiraz leaves at two developmental stages. These stages corresponded to berry pre- and post-veraison (20 and 96 days after flowering, respectively). The copy number of *VviLHY*, *VviGI* and *VviRVE1* had diel oscillations that were comparable to those occurring in pre-veraison berries (compare Fig. 1C and Fig. 6). These data suggest that the loss of rhythmicity of most clock genes post-veraison is not a whole-plant phenomenon and is potentially berry-specific.

Discussion

We identified that berry ripening affects the transcriptional profiles of circadian clock-associated genes in field-grown *Vitis vinifera*. We found that the amplitude of the oscillation of the majority of the transcripts decreased dramatically in berries after veraison (Fig. 7). In most cases, with the possible exception of *VviRVE1*, a recognisable oscillation was absent by the end of the ripening phase (Figs. 1C, 2, 4 and 5). At this developmental stage, the transcripts assumed a variety of average arrhythmic expression levels, with *VviELF4*, *VviLHY* and *VviRVE1* occupying a mid-point level, *VviPRR7a*, *VviGI*, *VviLIP1* and *VviPHYC* assuming a low level, and *VviPRR9* and *VviFKF1* having an abundance closer to the peak of expression at earlier developmental stages. *VviFKF1* transcripts accumulated to a greater abundance in Shiraz than Fiano.

The loss of rhythmicity of circadian clock-associated transcripts during berry ripening occurred in two grape cultivars, suggesting that this phenomenon is a general property of ripening grapes. This was not due to a general misregulation of transcript levels during berry ripening because, during the accumulation of ripening-associated transcripts increased during the ripening phase (Fig. 3, Supplemental Fig. 3). Experimentation with the two cultivars occurred during different seasons, suggesting that the rhythmicity changes were not due to unusual weather conditions for one experiment. Furthermore, the presence of the phenomenon in red (Shiraz) and white-skinned (Fiano) grape varieties suggests that the skin colour does not contribute to the process, which excludes the possibility that greater solar heating or altered photoperception in red cultivars underlies the arrhythmicity. The canopies of the two vines used also differed. The Fiano vines had open canopies, allowing considerable exposure to direct sunlight at certain times of the day, whilst the Shiraz canopies were much larger and, as a consequence, the Shiraz fruit was more extensively shaded. Despite this range of

differences, the changes to accumulation of the circadian clock-associated transcripts that occurred during berry ripening were similar in the two cultivars. Although many clock gene transcripts became arrhythmic during berry ripening, some (e.g. *ELF4, LHY, FKF1*) had greater abundance post-veraison at their times of lowest abundance pre-veraison (Fig. 1C). This likely explains why other studies report a general upregulation of circadian clock-associated transcripts post-veraison (Ghan et al. 2017, Tornielli et al. 2023), but time-series sampling is necessary to determine whether this is accompanied by a substantial reduction in amplitude or by arrhythmia.

Circadian clocks in different organs can operate with distinct characteristics. For example, in maize leaves approximately 23% of transcripts had a diel cycle of expression, compared with only 0.39% of transcripts in young ears (4-5cm in length) (Hayes et al. 2010). Furthermore, the transcripts that oscillate in both leaf and ear have much lower amplitudes of oscillation in the ear tissue compared with leaves, with many of these encoding core oscillator components (Hayes et al. 2010). Therefore, the differences between transcript cycling in grape leaves and berries appears to provide another example of a fundamental difference between vegetative and reproductive tissues (e.g. Fig. 1, Fig. 6).

There are a number of explanations for the dramatic changes in the amplitude of oscillation of circadian clock associated transcripts in post-veraison berries (Fig. 7). This might be a developmentally programmed phenomenon because berries are terminally-differentiated organs, or alternatively a secondary consequence of changes that occur during ripening. Under free running conditions, a transition to arrhythmia can indicate that individual cells are rhythmic but desynchronized from one another, so the sampling of many cells provides an average transcript abundance that appears arrhythmic (Paajanen et al. 2021, Muranaka and Oyama 2016). However, we think this unlikely within the post veraison berries, because under these circumstances the transcript abundance would be expected to assume the midpoint of the oscillation range. In contrast, the arrhythmic transcript abundance tends to assume a high, mid-point or low level, which could suggest oscillator arrest (Fig. 1C, Fig. 4). Furthermore, intercellular phase desynchronization tends to occur under free running conditions (Muranaka and Oyama 2016), whereas it seems less likely to occur under the cycles of entrainment cues (zeitgeber cycles) that occur under naturally fluctuating conditions.

The circadian clock in plants can respond to the metabolic state. In Arabidopsis, circadian oscillations are reinitiated in darkness by sucrose (Dalchau et al. 2011), and the expression of circadian oscillator components is affected by the concentration of sugars such that they can adjust the phase of the circadian oscillator (Bläsing et al. 2005, Haydon et al. 2013). This leads to a process whereby sugars produced by photosynthesis contribute to entrainment of the circadian oscillator, under certain conditions (Haydon et al. 2013, Frank et al. 2018). This is hypothesized to enhance the efficiency of primary metabolite usage over the day/night cycle (Webb et al. 2019). Whilst the metabolic environment of the ripening grape berry is very different from Arabidopsis leaves, it is possible that the high sugar environment interferes with signalling processes associated with circadian regulation to prevent dynamic cycling of circadian oscillator components. For

example, sugars can repress *PRR7* in Arabidopsis (Haydon et al. 2013), and in post-veraison grape berries *PRR7a* assumes low transcript abundance. Likewise, *PRR7* supresses *CCA1* in Arabidopsis, and the *CCA1* homologue *LHY* assumes a greater transcript abundance in post-veraison grape berries when *PRR7* levels are low. Whilst the concentrations of sugars in Arabidopsis leaves and post-veraison grape berries will differ by orders of magnitude (e.g. in Arabidopsis leaves, 1.5 – 4 µmol sucrose g fresh weight (Bläsing et al. 2005); in veraison grape berries 156, 143 and 3.27 µmol g fresh weight of glucose, fructose and sucrose, respectively (Wang et al. 2021)), it is also possible that differing calibration of sugar sensing pathways leads to similar outcomes in terms of oscillator dynamics.

A decrease in the amplitude of clock gene transcript cycling occurs in sweet chestnut (*Castanea sativa* Mill.), where the abundance of *CsTOC*, *CsLHY*, *CsPRR5*, *CsPRR7* and *CsPRR9* transcripts cycled in stems and leaves during summer, but not in buds and stems during winter dormancy (Ramos et al. 2005, Ibanez et al. 2008). Similarly, in whole Arabidopsis rosettes, under a light/dark regime low temperature reduced the amplitude of clock gene cycling and reduced the amplitude and rhythmicity of output genes (Bieniawska et al. 2008). There are some similarities between these effects of low temperature upon circadian clock transcript cycling and the changes that occur during ripening of grape berries. In the current study, air temperatures were not low during the ripening of Shiraz and Fiano berries (Supplemental Fig. 1, Supplemental Fig. 2). However, the rapid increase in hexose concentration in berries after veraison coincides with the loss of circadian gene cycling amplitude and rhythmicity. A feature shared between cold temperature stress and ripe berries with higher sugar content could be reduced water activity, which might be one explanation for why grape leaves do not have reduced amplitude of oscillation.

Changes to the behaviour of clock gene transcripts have been reported previously in grapes. Rienth et al. (2014) used the microvine to study diel changes in berry transcription at four developmental stages (two green and two ripening stages) at only two timepoints during the day/night cycle (two hours before the end of day, and two hours before the end of night). They suggested that the cycling of clock genes was more important in green berries than ripe berries. The authors proposed that the difference was due to photosynthesis in the skin of green berries, as opposed to ripe berries which have stored hexoses. However, they reported that *VviRVE1* (termed *VviCIR1* in their study) appeared to have a diel fluctuation at all developmental stages apart from the second post-veraison stage. Carbonell-Bejerano et al. (2014) studied diel oscillations of certain transcripts in near-ripe Tempranillo and Verdejo berries (19.3 and 21.1 ºBrix, respectively). They sampled at six times during a 24 h cycle, and found that of the clock genes, only *VviRVE1* had a diel fluctuation in all samples. These authors concluded that there might be a simplified clock in grape berries at later ripening stages. Our data show that the circadian clock transcripts oscillated as expected in berries before veraison, and the amplitude of expression of all clock genes decreased rapidly during the ripening phase (Figs. 1, 2, 4, and 5). *VviRVE1* continued to cycle until later stages of berry development than the other clock transcripts tested, but its diel rhythm did not appear to lead to oscillations in other components of the oscillator.

The changes in the amplitude of cycling of circadian clock transcripts at different stages of berry development provides a potent reminder that sampling timing and frequency is critical to accurately understand transcriptional dynamics and gene function during organ development. The time of measurement alone could determine whether a gene is thought to be expressed in a particular tissue or not, which may lead to misinterpretation of changes in expression levels. For example, a single dawn sample in pre- and post-veraison berries would suggest that *ELF4* transcript abundance increases after veraison, but the 24 h time-series indicates that *ELF4* transcript levels are generally lower post-veraison (Fig. 1C).

It is possible that changes during ripening in the diel cycling of certain clock genes might be part of the mechanisms that induce ripening or could form part of the wider suite of mechanisms that are necessary for ripening to proceed after it is induced. Alternatively, it could be a downstream consequence of ripening, rather than a regulator of this process. Considering the pervasive effect of the circadian oscillator upon transcriptome dynamics, the attenuation of oscillations of circadian clock components post-veraison might have a widespread effect upon gene transcription within ripening berries. This is because circadian clock components often have large numbers of regulatory targets, such as 439 (for CCA1), 722 (for LHY) and 772 (for TOC1) in Arabidopsis (Adams et al. 2018, Nagel et al. 2015, Gendron et al. 2012). Therefore, if the absence of diel cycles of circadian oscillator components in ripening berries translates into a loss of rhythmicity of circadian clock outputs, there could be downstream genome-wide changes in the transcriptional landscape (Fig. 7). This might explain the loss of diel fluctuations of many transcripts in a separate study (Rienth et al. 2014), although that study used only two timepoints so the data are difficult to interpret in terms of 24 h dynamics. It is generally thought that circadian clocks provide a selective advantage by aligning the phase of biological processes with the time in the environment (Dodd et al. 2005, Ouyang et al. 1998). Our finding that transcripts encoding some clock components stop cycling post-veraison could suggest that clock control is not necessary or even disadvantageous at later stages of berry ripening. For example, the shifts in circadian clock gene cycling might relate to changes in photosynthetic activity of grape berries. A recent proteomic study of chloroplasts in grape berry skin showed that the levels of proteins involved in the Calvin cycle are high pre-verasion but are much lower post-veraison, suggesting much less carbon fixation during the ripening phase (Teixeira et al. 2022). Parallels can be drawn between the later stages of fruit ripening and senescence processes. In Arabidopsis, the circadian clock might play a role in controlling ageing, with *PRR9* upregulating the expression of the positive regulator of senescence *ORE1* (Kim et al. 2018). In addition to the reduced amplitude of expression, we found that *VviPRR9* was expressed at a consistently high level in the later stages of berry ripening, which might indicate a regulatory role in berry senescence processes. The different functions of leaves (photosynthate exporters) and berries (terminally differentiated storage organs) may be responsible for the observed differences in circadian clock gene cycling. Although the mechanism that changes diel circadian clock cycling in ripening berries is unknown, we suggest that developmental changes in the ripening grape berry elicit alterations in diel circadian clock dynamics, which acts as a hub to drive changes in berry physiology.

Materials and Methods

Plant material and sampling strategy

Berries. Vines (*Vitis vinifera* L.) on own roots were sampled at a commercial vineyard (Chalk Hill Wines, Willunga, South Australia, latitude 35°15´S; longitude 138°33´E). During the 2010–2011 season, fruit from Shiraz vines (a red-skinned cultivar) were sampled at six stages of berry development (Table 1, Supplemental Table 1). The vines were spur pruned and trained in a double cordon structure. Drip irrigation was carried out throughout the season, including the sampling period. Commencing at 9am (ACDT — Australian Central Daylight Time), samples of 60 berries were obtained at four-hour intervals until the final sample at 9am on the following day. Berries were taken from all parts of bunches and from bunches located throughout the canopy to average out any shading effects. Three biological replicates comprising three adjacent vine rows were sampled at each time point. Sampling during the dark period occurred under very low light conditions. The vine rows were oriented in a North-South direction and berries were sampled from both sides of the canopy at each time point, with the berries from both sides combined to form a replicate. Berries were immediately frozen in liquid nitrogen and stored at -80°C. Additional sampling occurred as above during the 2018-2019 season for Fiano vines (a white-skinned cultivar) located at the same site, with vine rows also planted in a North to South orientation. These vines were sampled at two developmental stages, pre-veraison (PreV) and post-veraison (PostV) (Table 1, Supplemental Table 1). The frozen berries were deseeded, and the skin and flesh portion ground to a fine powder using an IKA A11 analytical mill (IKA, Staufen, Germany). Total soluble solids (TSS as degrees Brix) were measured using a RFM710 digital refractometer (Bellingham Stanley, Kent, UK) to provide a proxy of sugar content, because the hexoses glucose and fructose are the major contributing solutes during ripening.

Leaves. Mature (100-140 mm wide, from nodes 10-14) leaf samples were collected over a 24 h cycle during the 2019-2020 season from Shiraz vines, as described above for berries, except the vines were oriented in an East/West direction. These leaves were collected at 20- and 96-days post flowering (DPF). These timepoints are roughly equivalent to the sampling times for pre-veraison-I (PreV-I) and post-veraison-II (PostV-II) stages of Shiraz berry development respectively. The leaves showed no signs of senescence, degreening or fungal infection.

Hourly dry bulb air temperature data was obtained for the 24 h periods of berry sampling for the Shiraz and Fiano cultivars (Supplemental Figures 1 and 2). The data were sourced from the Bureau of Meteorology weather station at Noarlunga (station 23885, -35.16’S, 138.50’E approximately 15 km from the vineyard).

RNA isolation, cDNA synthesis and quantitative real-time PCR. Total RNA was isolated from grape berry tissue as described by Davies and Robinson (1996) and further purified as described by Symons et al. (2006). The same method was used for leaf tissue except the Tris-HCl in the extraction buffer was pH 7.5 rather than pH 8.3 for berries. First-strand cDNA for quantitative real-time PCR (qRT-PCR) was synthesized using the

Transcriptor First Strand cDNA synthesis Kit as per manufacturer's instructions (Roche, Mannheim, Germany), using 1 µg of total RNA and the anchored oligo(dT)18 primer. The cDNA was diluted to 400 µL with water. Quantitative RT-PCR was conducted using a Roche Lightcycler 489 (Roche). Reactions of 7.5 µL containing 3.75 µL of LightCycler 480 SYBR Green I Master Mix (Roche) with 2.5 µL of cDNA and the two primers at a final concentration of 0.5 µM were done in triplicate. The cycles were an initial cycle of 95°C for 5 minutes followed by 45 cycles 95°C for 20 seconds, 58°C for 20 seconds, 72°C for 20 seconds with a final extension of 5 minutes. The melt curve was conducted from 50 to 95°C. The gene-specific primer pair for the reference gene used for cDNA normalisation, *ACT2*, has been published previously (Böttcher et al. 2011) and the primers used for the analysis of the expression of the other genes are given in Supplemental Table 3. Each PCR was performed in triplicate. To calculate the copy number of the transcripts in each reaction, the purified gene fragments used for the standard curves were quantified using PicoGreen (AGRF, Adelaide, South Australia) and the number of molecules in each standard dilution was determined according to Whelan et al. (2003). The specificity of the reactions was confirmed by melt curve analysis as well as separation on agarose gels. The identity of each product was confirmed by DNA sequencing (AGRF, Adelaide, South Australia). The significance of changes in expression data was determined by ANOVA, followed by Duncan's post hoc test using IBM SPSS software (ver. 20; IBM Australia, Sydney, NSW, Australia).

Data availability statement

Loci of genes used for RT-qPCR analysis and the corresponding primers sequences are given in Supplemental Table 3.

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Author Contributions

C.D. (rasdavies004@gmail.com), designed the research, conducted the field studies, contributed to data analysis, data interpretation and figure preparation, and co-authored the paper; C.A.B. (crista.burbidge@csiro.au), conducted gene expression analysis, contributed to data analysis and figure

preparation; C.B. (christine.bottcher@csiro.au), contributed to data analysis and co-authored the paper; A.D. (antony.dodd@jic.ac.uk), contributed to data analysis, data interpretation, figure preparation, and co-authored the paper.

Disclosures

The authors have no conflict of interest to declare.

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608

609 **Tables**

610 **Table 1.** Berry developmental stage, as defined by Brix and days post flowering

611

Berry developmental stage	**Days post-flowering***	**ºBrix**	
		Mean	SE
Shiraz			
Pre-veraison-I	30	4.69	0.04
Pre-veraison-II	43	5.26	0.04
Veraison	57	5.70	0.05
Post-veraison-I	79	16.17	0.15
Post-veraison-II	93	19.40	0.13
Post-veraison-III	107	23.35	0.14
Fiano			
Pre-veraison	44	4.33	0.04
Post-veraison	105	21.64	0.07

612

613 *Number of days from 50% flowering to commencement of 24 h sampling period

614

615

Legends

Tables

Table 1. Berry developmental stage, as defined by Brix and days post flowering

Figures

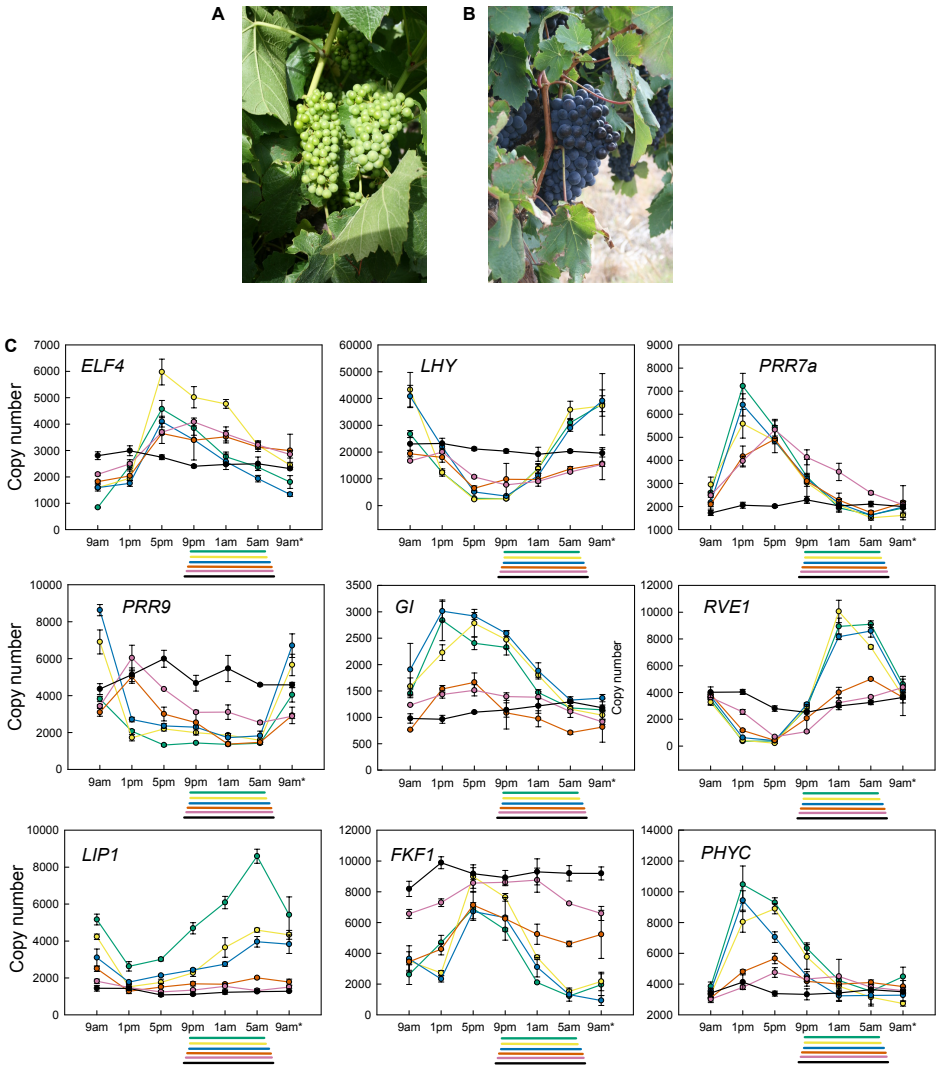
Figure 1. Diel cycling of putative circadian clock and clock output genes transcripts changed significantly during berry ripening. A) Pre-veraison (PreV-II stage) Shiraz berries and B) post veraison (PostV-II stage) Shiraz berries. C) Transcript abundance in grape berries of circadian clock-associated genes, over a 24 h cycle. Data shown for six stages during Shiraz (red) grape berry development represent means of three biological replicates +/- SE. The line colours and symbols indicate the six developmental stages at which transcript abundance was measured: green = PreV-I, yellow = PreV-II, blue = Veraison, brown = PostV-I, pink = PostV-II, black = PostV-III. The coloured lines below the X-axes indicate the time of the period between sunset and sunrise (Supplemental Table 1), with the line colour corresponding to the developmental stage, in order of developmental stage from top (green, PreV-I) to bottom (black, PostV-III).

Figure 2. The amplitude of diel cycling of circadian clock-related genes decreased during berry ripening. The amplitude of putative circadian clock and clock output genes is shown throughout Shiraz (red) berry development (PreV-I to PostV-III). All data represent means of three biological replicates +/- SE. Different letters indicate that the means differ significantly ($P<0.05$ using one-way ANOVA followed by Duncan's post-hoc test).

Figure 3. **Transcription processes remained active throughout berry ripening.** Diel changes in expression of non-circadian clock genes (*VviTL1, VviCSLG2, VviIAA19*) were observed at six stages during Shiraz (red) grape berry development. All data represent means of three biological replicates +/- SE. The line colours and symbols indicate the six developmental stages at which transcript abundance was measured: green = PreV-I, yellow = PreV-II, blue = Veraison, brown = PostV-I, pink = PostV-II, black = PostV-III. The coloured lines below the X-axes indicate the time of the period between sunset and sunrise (Supplemental Table 1), with the line colour corresponding to the developmental stage, in order of developmental stage from top (green, PreV-I) to bottom (black, PostV-III).

Figure 4. Altered diel cycling of expression of putative circadian clock and clock output genes was not limited to a single cultivar or season. The diel expression patterns in Fiano (white) grape berries are shown at developmental stages equivalent to Shiraz stages PreV-II and PostV-III (Table 1). All data represent means of three biological replicates +/- SE. The line colours and symbols indicate the developmental stage at which gene expression was measured: green = PreV-II, black = PostV-III. Coloured lines below the X-axis indicate the period between sunset and sunrise (Supplemental Table 1), with line colour corresponding to the developmental

652 stage. Different letters indicate that the means differ significantly (P<0.05 using one-way ANOVA followed by
653 Duncan's post-hoc test; N.S. = not significant).
654
655 **Figure 5. The amplitude of diel cycling of circadian clock-related genes also decreased during Fiano (white**
656 **grape) berry ripening.** The estimated amplitude of putative circadian clock and clock output genes are shown
657 for two stages berry development: Pre = PreV-II, Post = PostV-III. All data represent means of three biological
658 replicates +/- SE. *VviLHY* and *VviLIP1* had unequal variance as determined by F-test, so the Welch test was used
659 for these, and t-tests used for the other genes. * = significant in one-tailed t-test at P<0.05, ** = significant at
660 P<0.005.
661
662 **Figure 6. Cycling of circadian clock-related gene transcripts continued in mature leaves pre-and post-**
663 **veraison.** Diel changes in expression are shown for putative circadian clock and clock output genes in mature
664 Shiraz leaves on two dates corresponding to pre-veraison and post-veraison stages of Shiraz grape
665 development. Data represent the means of three biological replicates +/- SE. The line colours and symbols
666 indicate the developmental stage at which gene expression was measured, blue = PreV; black = PostV.
667 Coloured lines below the X-axis indicate the period between sunset and sunrise (Supplemental Table 1); blue =
668 PreV, black = PostV.
669
670 **Figure 7. Transition in circadian oscillator dynamics during grape berry ripening.** Pre-veraison, when berry
671 hexose content is low, circadian clock transcripts in developing grape berries and leaves oscillate with high
672 amplitude. Post-veraison, when berry hexose content is high, circadian oscillator transcripts in grape berries
673 oscillate with very low amplitude or are arrhythmic, although the berries remain transcriptionally active. In
674 contrast, circadian clock transcripts in leaves continue to oscillate post-veraison.
675
676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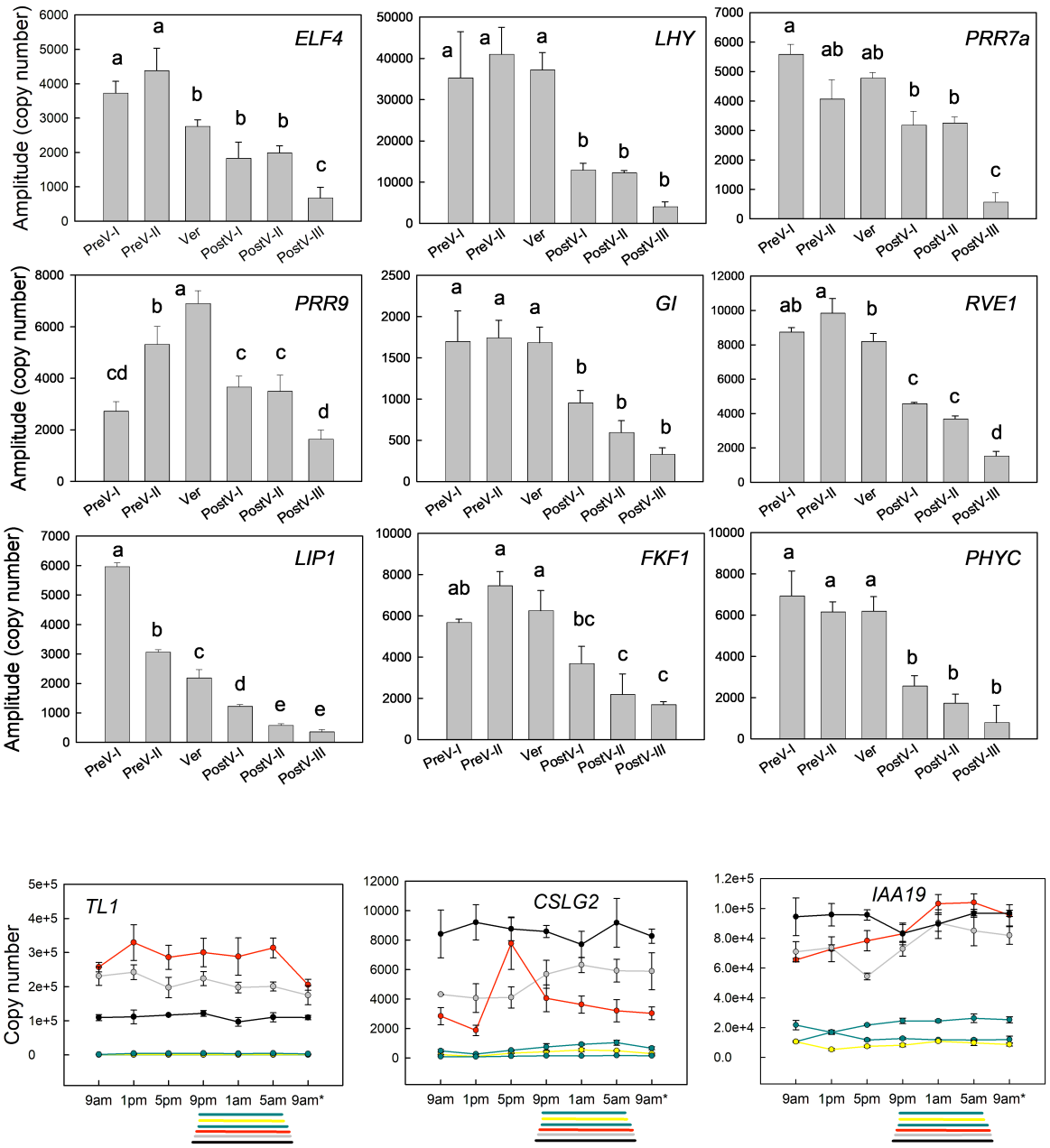
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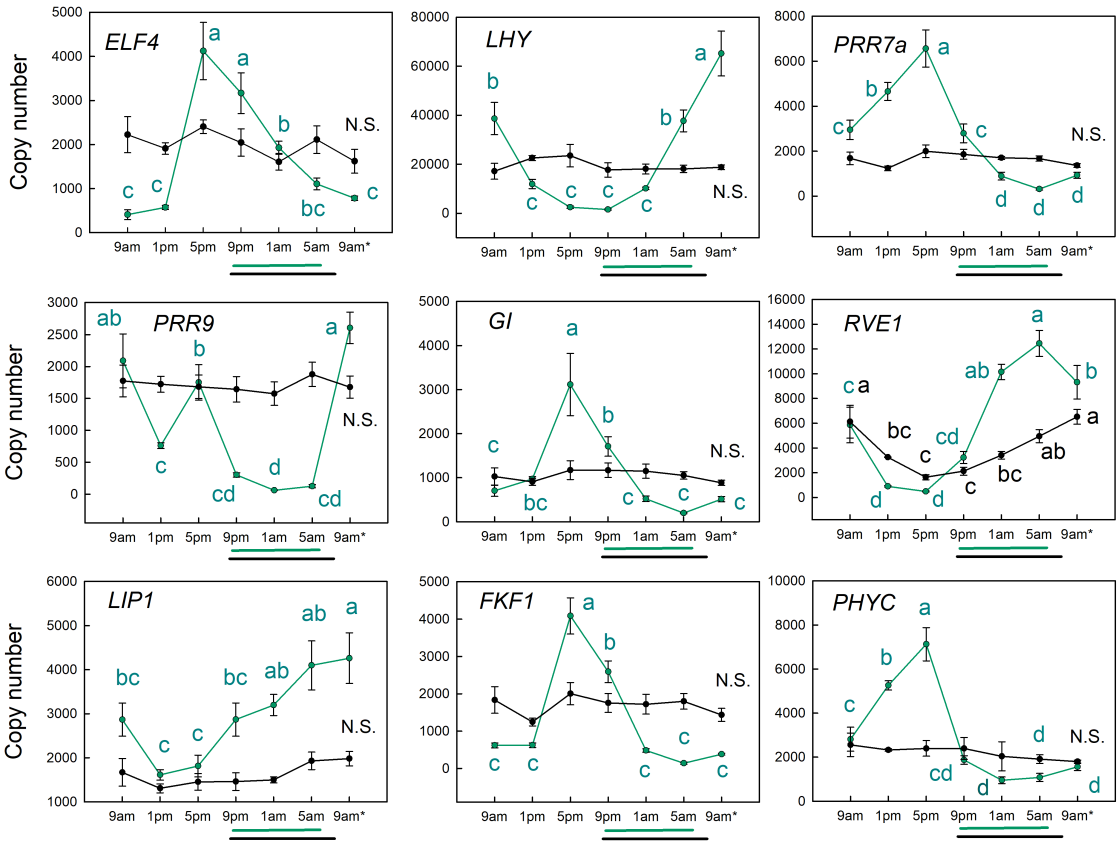
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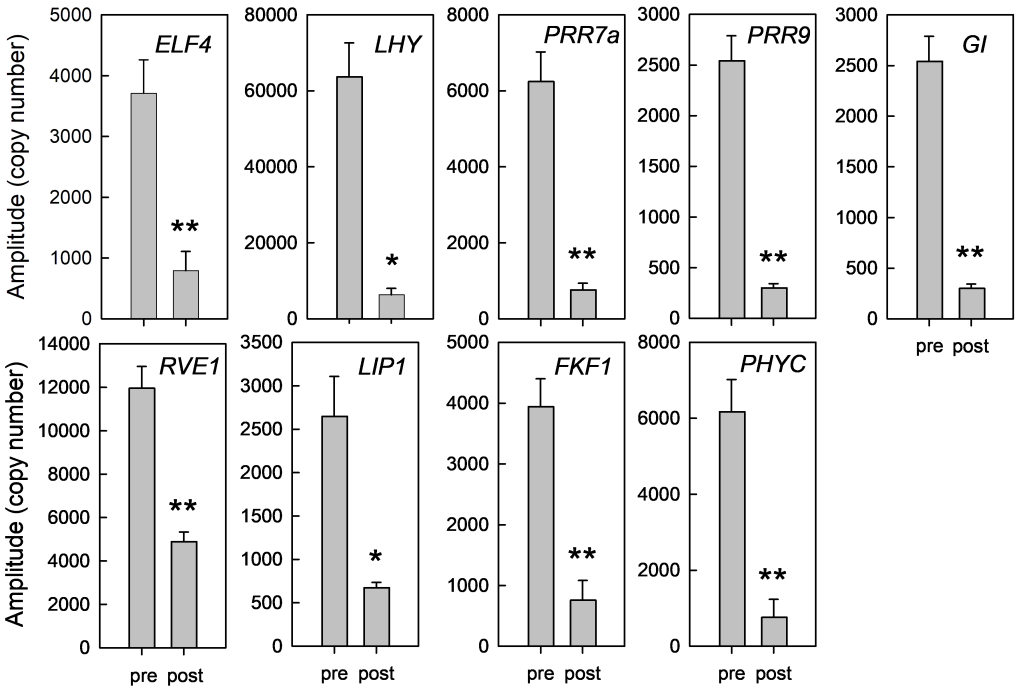
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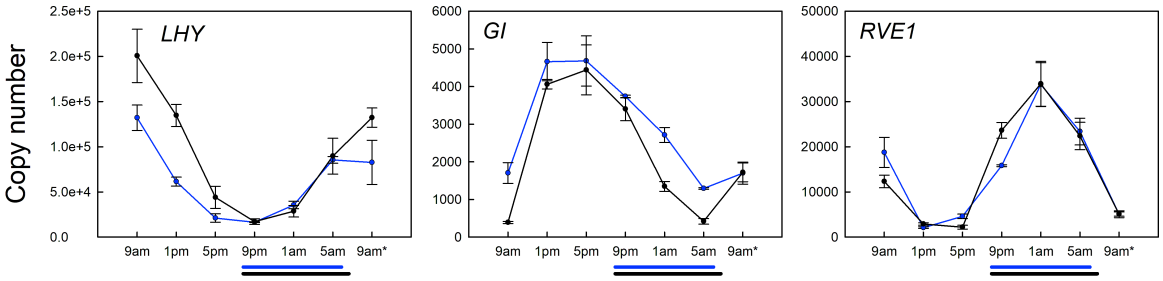
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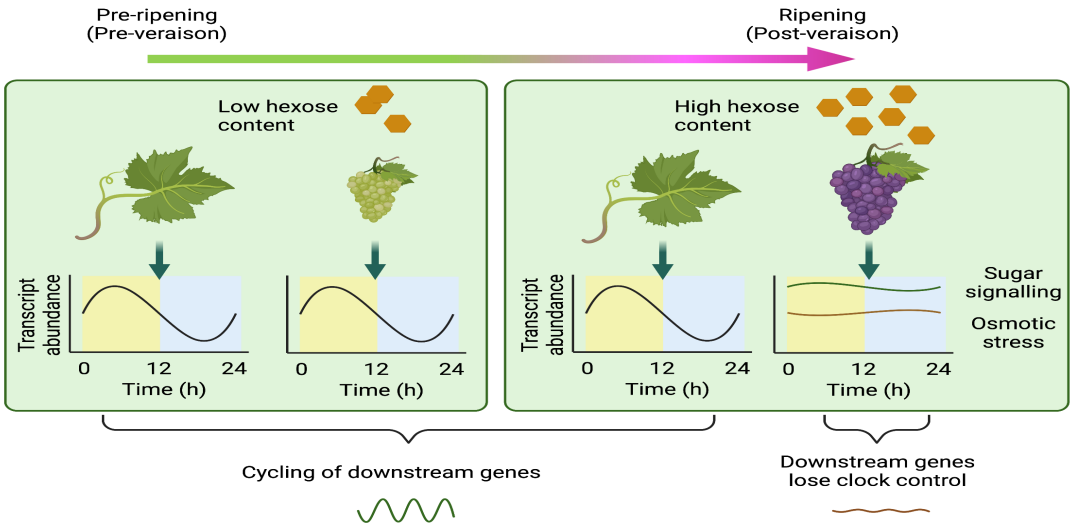

677 **Supplemental data**

678

679 **Supplemental Table 1.** Berry and leaf sampling dates, sunrise/sunset times for each time-
680 series. Sunrise and sunset times were derived for latitude 35°15'S, longitude 138°33'E using
681 information from http://www.ga.gov.au/geodesy/astro/sunrise.jsp with a UTC time offset of
682 +10.5 h

683

Developmental stage	Date of sampling	Sunrise time	Sunset time
Shiraz berries			
Pre-veraison-I	22/12/2010	05:58	20:30

	23/12/2010	05:58	20:30
Pre-veraison-II	4/01/2011	06:07	20:34
	5/01/2011	06:07	20:34
Veraison	18/01/2011	06:19	20:32
	19/01/2011	06:20	20:32
Post-veraison-I	9/02/2011	06:42	20:17
	10/02/2011	06:43	20:16
Post-veraison-II	24/02/2011	06:57	20:01
	25/02/2011	06:58	19:59
Post-veraison-III	10/03/2011	07:10	19:42
	11/03/2011	07:11	19:41
Fiano berries			
Pre-veraison-II	20/12/2018	05:57	20:29
	21/12/2018	05:57	20:29
Post-veraison-III	20/02/2019	06:53	20:05
	21/02/2019	06:54	20:04
Shiraz leaf			
20 DPF ('Pre-veraison')	28/11/2019	05:55	20:12
	29/11/2019	05:55	20:13
96 DPF ('Post-veraison')	12/02/2020	06:45	20:15
	13/02/2020	06:46	20:13

684

685

686 **Supplemental Table 2.** Loci of genes used in qRT-PCR analysis of transcript abundance,
687 with putative functions listed. [a]Genes identified as per the Grape Gene Reference Catalogue
688 (http://www.vitviz.tk/Catalogue/, Navarro-Paya et al., 2022), [b]Primers designed to mRNA sequence
689

Locus ID	**Gene Symbol**	**Name in *Arabidopsis***	**Putative function**
Vitvi15g00870	*VviLHY*[a]	LATE ELONGATED HYPOCOTYL	myb-related transcription factor involved in circadian rhythm
Vitvi13g01892	*VviELF4*[a]	EARLY FLOWERING 4	phytochrome-regulated component of a negative feedback loop involving circadian clock central oscillator components
Vitvi06g00368	*VviPRR7a*[a]	PSEUDO RESPONSE REGULATOR 7	member of temperature-sensitive circadian system, transcriptional repressor
Vitvi15g00879	*VviPRR9*[a]	PSEUDO RESPONSE REGULATOR 9	member of temperature-sensitive circadian system, transcriptional repressor
Vitvi18g01553	*VviGI*[a]	GIGANTEA	involved in many processes including circadian clock control
Vitvi07g01846	*VviLIP1*	LIGHT INSENSITIVE PERIOD 1	ras-related small gtp-binding family, regulates light input into the circadian clock
Vitvi04g00845	*VviRVE1*[a]	REVEILLE 1	Myb-like protein that promotes circadian clock pace
Vitvi01g00499	*VviFKF1*	FLAVIN-BINDING, KELCH REPEAT, F-BOX1	circadian clock-associated protein
Vitvi12g00715	*VviPHYC*	PHYTOCHROME C	regulatory photoreceptor
Non-circadian genes			
NM_001281132.2[b]	*VviTL1*	THAUMATIN-LIKE PROTEIN 1	response to biotic and abiotic stresses

Vitvi05g00676	*VviCSLG2*	CELLULOSE SYNTHASE-LIKE PROTEIN G2-LIKE	cell wall synthesis, product unknown
Vitvi09g00436	*VviIAA19*	AUX/IAA TRANSCRIPTION FACTOR	repressor of auxin-related transcription

690

691

692 **Supplemental Table 3.** Primers used for RT-qPCR analysis

693

Gene ID	Gene symbol	Forward Primer 5'-3'	Reverse Primer 5'-3'	No. of splice variants	No. detected by primers
Vitvi15g00870	*VviLHY*	AAGGGGACGCTTCAACCTGA	TGTGCAAGGGGAAGGGGA	1	1
Vitvi13g01892	*VviELF4*	ACGAGAACCACCAGTCCAAGA	CCGTTTTCGTTCTCGTTTGGC	3	3
Vitvi06g00368	*VviPRR7a*	GGTTGAAGGTTGCTCGCTTGT	TTTGGTAAGAGAGCACATGGCC	7	7
Vitvi15g00879	*VviPRR9*	CCTTTGTGCCTGATGGTGAAT	GCCCTAAACACCCTACCCAGA	4	4
Vitvi18g01553	*VviGI*	TGCCCAGGTTAGTTTCTTCTGTT	GATGCTACCACCTGATTTTGCC	7	7
Vitvi07g01846	*VviLIP1*	ATGGGGTGAAGCCATACAATGT	CCCACAACCATGAATGAATCAA	6	6
Vitvi04g00845	*VviRVE1*	GTTTGGCAGAGAGAGACACC	GCGCTCTAGGGGATTTCTTT	1	1
Vitvi01g00499	*VviFKF1*	ACAGCACCTGTGTGGTTGGA	CTGCAGTGTGGGCAACTCAC	1	1
Vitvi12g00715	*VviPHYC*	CATGGCTTCCCACCTTCTGTAC	TCACTTGCCTGGCACTGTTTC	2	1
NM_001281132.2	*VviTL1*	AGAGTACAAGACACCCAGGA	TATCACATCACATGCGCACA	1	1
Vitvi05g00676	*VviCSLG2*	AAGGACAAGGGTCGCATTCC	AGCTCAATACAGCAGAACAACA	1	1
Vitvi09g00436	*VviIAA19*	GATCGATTTGAGTTCCCACCAAG	TCTTTCAATGCTTCACCGATGC	2	1

694

695

Supplemental Table 4. Statistical significance of changes in transcript expression determined by ANOVA followed by Duncan's post hoc test. Different letters indicate that the means differ significantly ($P < 0.05$). N.S. = not significant.

Gene	Stage	Time						
		9am	**1pm**	**5pm**	**9pm**	**1am**	**5am**	**9am***
VviELF4	PreV-I	e	cd	a	b	c	cd	d
	PreV-II	e	de	a	b	b	c	cd
	Ver	de	de	a	b	c	d	e
	PostV-I	c	bc	a	ab	a	abc	abc
	PostV-II	e	de	ab	a	ab	bc	cd
	PostV-III	ab	a	abc	bc	bc	bc	c
VviLHY	PreV-I	ab	c	c	c	bc	a	a
	PreV-II	a	bc	c	c	b	a	a
	Ver	a	b	c	c	c	b	a
	PostV-I	a	ab	b	ab	ab	ab	ab
	PostV-II	b	a	d	e	e	c	b
	PostV-III	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*
VviPRR7a	PreV-I	cd	a	b	c	de	d	de
	PreV-II	bc	a	a	b	cd	d	d
	Ver	d	a	b	c	d	d	d
	PostV-I	c	ab	a	bc	c	c	c
	PostV-II	c	b	a	b	b	c	c
	PostV-III	b	ab	ab	a	ab	ab	ab
VviPRR9	PreV-I	a	b	c	c	c	c	a
	PreV-II	a	c	c	c	c	c	b
	Ver	a	c	c	c	c	c	b
	PostV-I	b	a	b	bc	c	c	b
	PostV-II	bc	a	b	c	c	c	c
	PostV-III	b	ab	a	b	ab	b	b

VviGI	PreV-I	b	a	a	a	b	b	b
	PreV-II	de	bc	a	ab	cd	ef	f
	Ver	b	a	a	a	b	b	b
	PostV-I	c	ab	a	abc	bc	c	c
	PostV-II	bc	ab	a	ab	ab	cd	d
	PostV-III	b	b	ab	ab	ab	a	ab
VviRVE1	PreV-I	bc	d	d	c	a	a	b
	PreV-II	c	d	d	c	a	b	c
	Ver	bc	d	d	c	a	a	b
	PostV-I	ab	c	c	bc	ab	a	ab
	PostV-II	b	c	d	d	b	b	a
	PostV-III	a	a	bc	c	bc	abc	ab
VviLIP1	PreV-I	b	c	c	b	b	a	b
	PreV-II	ab	c	c	c	b	a	ab
	Ver	ab	d	cd	bcd	bc	a	a
	PostV-I	a	d	cd	bcd	bcd	b	bc
	PostV-II	a	ab	b	b	ab	b	ab
	PostV-III	a	a	b	b	ab	ab	ab
VviFKF1	PreV-I	c	b	a	b	cd	d	cd
	PreV-II	bc	bcd	a	a	b	d	cd
	Ver	b	bc	a	a	b	c	c
	PostV-I	b	ab	a	ab	ab	ab	ab
	PostV-II	c	bc	ab	ab	a	bc	c
	PostV-III	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*
VviPHYC	PreV-I	c	a	a	b	c	c	c
	PreV-II	c	a	a	b	c	c	c
	Ver	c	a	b	c	c	c	c
	PostV-I	c	ab	a	b	bc	b	bc

	PostV-II	b	ab	a	ab	ab	ab	ab
	PostV-III	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*

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703 **Supplemental Figures**

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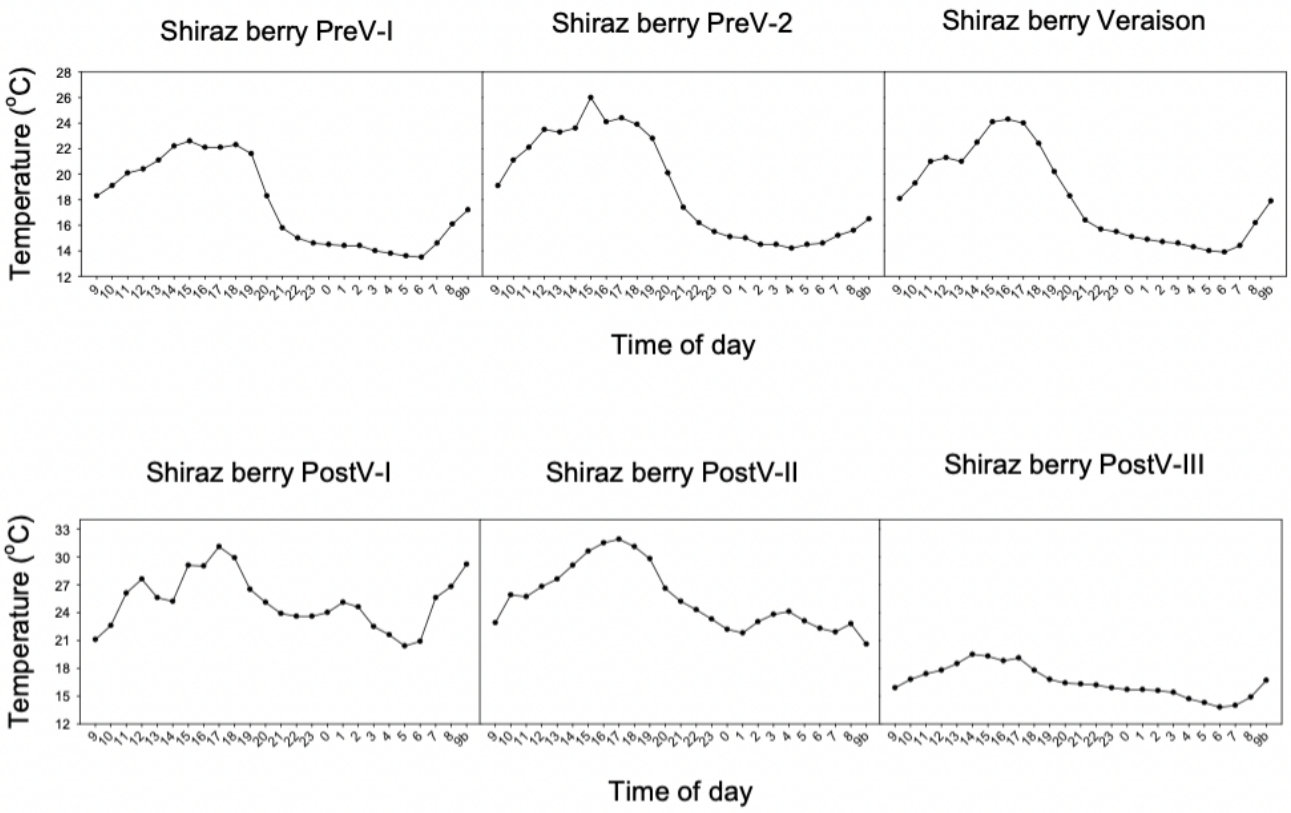

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707 **Supplemental Figure 1** Hourly dry-bulb measurements of air temperature for the six 24 h
708 sampling periods for Shiraz (red) berries, dates as per Supplemental Table 1. Numbers on
709 the X-axis indicate time of day in hours.

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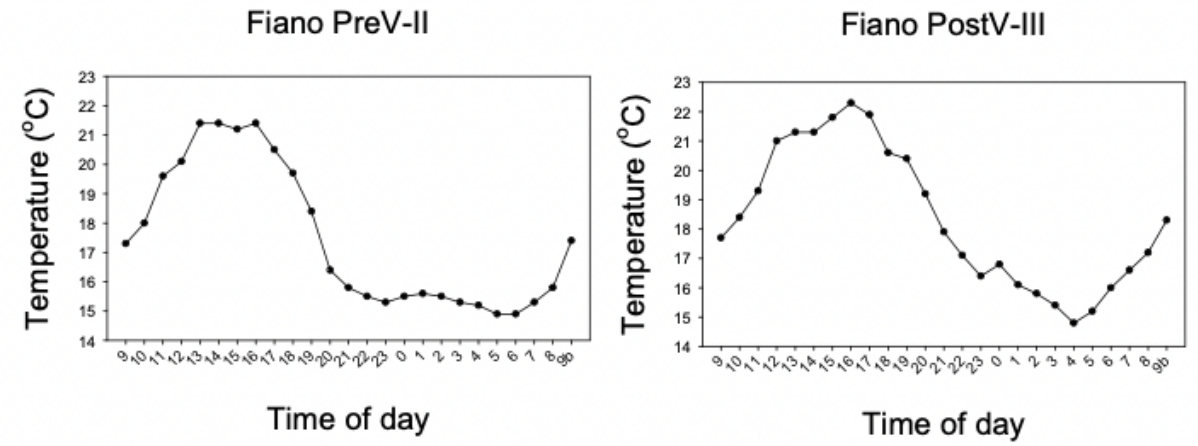
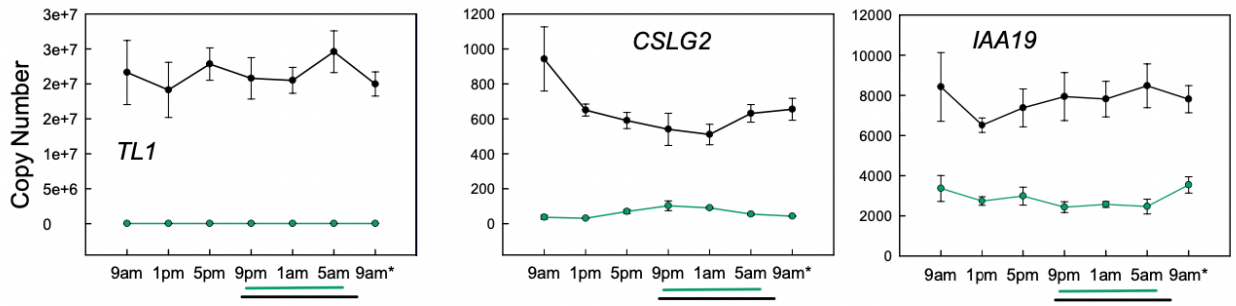


Supplemental Figure 2 Hourly, dry bulb measurements of air temperature for the two 24 h sampling periods for Fiano (white) berries, dates as per Supplemental Table 1. Numbers on the X-axes indicate time of day in hours.



Supplemental Figure 3 Transcription processes remained active throughout Fiano berry ripening. Diel changes in expression amplitude of non-circadian clock genes are shown for two stages of Fiano berry development: green line and symbols = PreV-II stage; black line and symbols = PostV-III stage. All data represent means of three biological replicates +/- SE. Coloured lines below the X-axis indicate the period between sunset and sunrise (Supplemental Table 1), with the line colour corresponding to the developmental stage.