A significant role for the circadian clock in the long-term water use efficiency of Arabidopsis

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Word count (body text including methods): 5374

Short title: Circadian regulation and water use efficiency

Author contributions: N.M.L.S. and A.N.D. conceived experiments, N.M.L.S. and N.E.C. performed experiments, N.M.L.S., A.M.H. and A.N.D. analysed and interpreted the data, N.M.L.S., A.M.H. and A.N.D. wrote the paper. A.N.D. agrees to serve as the author responsible for contact and ensures communication.

One-sentence summary: The circadian clock in Arabidopsis makes an important contribution to long-term water use efficiency.
Abstract

In plants, water use efficiency is a complex trait derived from numerous physiological and developmental characteristics. Here, we investigated the involvement of circadian regulation in long-term water use efficiency. Circadian rhythms are generated by the circadian oscillator, which provides a cellular measure of the time of day. In plants, the circadian oscillator contributes to the regulation of many aspects of physiology, including stomatal opening, the rate of photosynthesis, carbohydrate metabolism and developmental processes. We investigated in Arabidopsis the impact upon whole plant, long-term water use efficiency of the misregulation of genes encoding a large number of components of the circadian oscillator, identifying a major role for the circadian oscillator in plant water use. This appears to be due to contributions of the circadian clock to the control of transpiration and biomass accumulation. We also identified that the circadian oscillator specifically within guard cells contributes to both long-term water use efficiency and dehydration tolerance. Our experiments indicate that knowledge of circadian regulation will be important for developing future crops that use less water.

Introduction

World population growth is increasing the demand for fresh water for agriculture, with climate change predicted to exacerbate this competition for water resources (Ruggiero et al., 2017). One strategy to sustainably increase agricultural production involves the improvement of crop water use (Condon et al., 2004; Xoconostle-Cazares et al., 2010; Hu and Xiong, 2014; Ruggiero et al., 2017). Since up to 97% of water taken up from the soil by plants is lost through stomatal transpiration (Yoo et al., 2009; Na and Metzger, 2014), the manipulation of transpiration represents an excellent candidate for designing crops with increased water use efficiency.
Plant water loss can be manipulated through changes in the regulation of stomatal opening and by altering stomatal density and patterning (Pei et al., 1998; Hugouvieux et al., 2001; Schroeder et al., 2001; Hetherington and Woodward, 2003; Yoo et al., 2010; Lawson and Blatt, 2014; Franks et al., 2015; Caine et al., 2019). In addition to stomatal responses to environmental cues such as light, temperature and phytohormones, there are circadian rhythms of stomatal opening (Gorton et al., 1989; Hennessey and Field, 1991). Circadian rhythms are self-sustaining biological cycles with a period of about 24 h. These rhythms are thought to adapt plants to daily cycles of light and dark, by anticipating daily changes in the environment and co-ordinating cellular processes. In higher plants, circadian rhythms are generated by several interlocked transcription-translation feedback loops known as the circadian oscillator (Hsu and Harmer, 2014). The phase of the circadian oscillator is adjusted continuously to match the phase of the environment through the process of entrainment, in response to light, temperature and metabolic cues (Somers et al., 1998; Millar, 2004; Salomé and McClung, 2005; Haydon et al., 2013). Additionally, the circadian oscillator communicates an estimate of the time of day to circadian-regulated features of the cell, initially through transcriptional regulation (Harmer et al., 2000). The known circadian oscillator controls circadian rhythms of stomatal opening because mutations that alter the circadian period or cause circadian arrhythmia lead to equivalent alterations in the circadian rhythm of stomatal opening (Somers et al., 1998; Dodd et al., 2004; Dodd et al., 2005). The circadian oscillator is also involved in the responses of guard cells to environmental cues such as drought and low temperature (Dodd et al., 2006; Legnaioli et al., 2009).

Circadian rhythms are often studied under conditions of constant light. However, the circadian oscillator is also important for the regulation of stomatal opening under cycles of light and dark. For example, overexpression of the circadian oscillator component CCA1 (CCA1-ox) alters the daily regulation of stomatal opening such that stomatal conductance increases steadily throughout the photoperiod (Dodd et al., 2005). In comparison, in wild type plants stomatal conductance remains relatively uniform during the photoperiod and is
substantially lower than CCA1-ox (Dodd et al., 2005). This suggests that misregulation of the
circadian oscillator might alter plant water use under cycles of light and dark.
Overexpression of CCA1 specifically within guard cells, using a guard cell specific promoter,
alters flowering time and drought response phenotypes under cycles of light and dark
(Hassidim et al., 2017). Like constitutive CCA1 overexpression (Dodd et al., 2005), CCA1
overexpression specifically within guard cells generally causes greater stomatal opening
during the light period (Hassidim et al., 2017). Therefore, the circadian oscillator within guard
cells is important for the daily regulation of stomatal opening (Hassidim et al., 2017).
Modelling suggests that under light/dark cycles, the circadian oscillator contributes at the
 canopy scale to daily rhythms in stomatal aperture and carbon assimilation in bean and
cotton (Resco de Dios et al., 2016).
The contribution of the circadian oscillator to both stomatal opening and growth and biomass
accumulation (Dodd et al., 2005; Graf et al., 2010) suggests that the circadian oscillator
might make an important contribution to water use efficiency (WUE). WUE is the ratio of
carbon dioxide incorporated through photosynthesis into biomass to the amount of water lost
through transpiration. At the single leaf level, instantaneous, intrinsic WUE is often
measured with gas exchange techniques and expressed as net CO₂ assimilation per unit of
water transpired (Vialet-Chabrand et al., 2016; Ruggiero et al., 2017; Ferguson et al., 2018).
However, such measurements do not provide an accurate representation of WUE over the
plant lifetime, which is influenced by features such as leaf position, dark respiration, and time
of day changes in instantaneous WUE (Condon et al., 2004; Tomás et al., 2014; Medrano et
al., 2015; Ferguson et al., 2018). It is important to note that WUE is not a drought resistance
trait (Blum, 2009).
Given that the circadian oscillator affects stomatal opening and biomass accumulation
(Gorton et al., 1989; Hennessey and Field, 1991; Dodd et al., 2005; Edwards and Weinig,
2010; Graf et al., 2010; Edwards et al., 2012), we hypothesized that specific components of
the circadian oscillator might make an important contribution to long-term WUE of plants.
Therefore, we investigated the impact of the misregulation of parts of the circadian oscillator upon the long-term WUE of Arabidopsis. We identified that the circadian oscillator has profound effects upon the long-term WUE of plants. Importantly, some alterations in oscillator function increase long-term WUE, suggesting potential targets for future improvements of crop WUE.

Results

Circadian oscillator components contribute to water use efficiency

Each background accession had a distinct WUE (C24: 3.01 ± 0.07 mg ml\(^{-1}\); Col-0: 2.22 ± 0.02 mg ml\(^{-1}\); L. er.: 1.60 ± 0.04 mg ml\(^{-1}\); Ws: 1.91 ± 0.06 mg ml\(^{-1}\)) (Fig. S1). These differences between backgrounds are consistent with previous studies of WUE, stomatal function and stomatal density in Arabidopsis (Nienhuis et al., 1994; Woodward et al., 2002; Dodd et al., 2004; Masle et al., 2005; Karaba et al., 2007; Ruggiero et al., 2017; Ferguson et al., 2018).

We identified that correct regulation of the circadian oscillator makes a substantial contribution to WUE. 33 single mutants or overexpressors of genes associated with circadian regulation, representing 22 circadian oscillator-associated components, were screened for WUE (Fig. 1). Nearly half of the mutants or overexpressors examined had a significantly different WUE from the wild type \((p < 0.05; 16\) of 33 genotypes). This corresponded to mutants or overexpressors representing half of the circadian oscillator components covered by our study \(11\) of 22 genes \(\) (Fig. 1). The \(cca1\)-11, \(elf3\)-1, \(prr5\)-3, \(prr9\)-1, \(tps1\)-11, \(tps1\)-12, and \(ztl\)-1 mutants, as well as the \(TOC1\) and \(KIN10\) (line 6.5) overexpressors, had significantly lower WUE than the wild type \(\) (Fig. 1). The \(gi\)-2, \(gi\)-11, \(grp7\)-1, \(prr7\)-11 and \(tej\)-1 mutants had significantly greater WUE than the wild type \(\) (Fig. 1). This suggests that misregulating the expression of circadian clock components \(CCA1, ELF3, GI, GRP7, PRR5, PRR7, PRR9, TEJ, TOC1\) and \(ZTL\) changes whole plant long-term WUE \(\) (Fig. 1). \(tic\)-2 had significantly greater WUE than the wild type, whereas \(tic\)-1 had significantly lower WUE than the wild type, so the effect of \(TIC\) mutation upon WUE is
unclear (Fig. 1). We also included the che-2 mutant in our initial analysis, but inconsistency of its WUE phenotype between experimental repeats led us to exclude the data. WUE was also altered by changing the expression of the energy signalling components TPS1 and KIN10 that participate in inputs to the circadian oscillator (Shin et al., 2017; Frank et al., 2018) (Fig. 1). Therefore, correct expression of these circadian clock-associated genes contributes to long-term WUE of Arabidopsis.

We were interested to determine whether the WUE alterations caused by misregulation of circadian oscillator gene expression arose from changes in either biomass accumulation or transpiration. There was no clear evidence that a change in one of these parameters alone underlies the altered WUE phenotypes (Fig. 2A). This suggests that the altered WUE of lines with misregulated circadian clock genes is due to the net effect of altered biomass accumulation and altered transpiration in these genotypes (Fig. 2A).

We hypothesised that variations in WUE might be explained by specific circadian phenotypes in the mutants and overexpressors that we tested. For example, mutations in clock genes expressed with a particular set of phases might have a pronounced effect on WUE. Likewise, the nature of the circadian period change or flowering time change resulting from misexpression of each oscillator component might be associated with certain changes in WUE. To test this, we related the data from our WUE screen to the circadian phase of expression of each mutated or overexpressed gene. We also compared the direction of change of WUE to the period and flowering time phenotypes that arise from each mutant or overexpressor (Fowler et al., 1999; Schultz et al., 2001; Doyle et al., 2002; Nakamichi et al., 2002; Yanovsky and Kay, 2002; Imaizumi et al., 2003; Más et al., 2003; Murakami et al., 2004; Farré et al., 2005; Hazen et al., 2005; Baena-González et al., 2007; Streitner et al., 2008; Wang et al., 2008; Baudry et al., 2010; Nakamichi et al., 2010; Rawat et al., 2011; Wahl et al., 2013; Hsu and Harmer, 2014). We note that the phenotypes reported by these studies were often identified under constant conditions, whereas our experiments occurred under light/dark cycles.
There was no obvious relationship between the circadian phenotypes that are caused by each mutant or overexpressor investigated and the WUE of each of these lines (Fig. 2B, C, D). For example, mutating morning-phased circadian oscillator components can either decrease or increase WUE (Fig. 2B). Mutants that cause long circadian periods and short circadian periods can both increase and decrease WUE (Fig. 2C). Furthermore, mutants and overexpressors that cause both early and delayed flowering can each increase and decrease WUE (Fig. 2D).

Circadian regulation of water use efficiency combines multiple traits

Mutation or overexpression of components of the circadian oscillator can cause changes in the development of Arabidopsis, such as alterations in rosette size, leaf shape and petiole length (Fig. 3A) (Zagotta et al., 1992; Schaffer et al., 1998; Wang and Tobin, 1998; Dodd et al., 2005; Ruts et al., 2012; Rubin et al., 2018). These changes are likely to have implications for gas exchange because, for example, spatially separated leaves are predicted to transpire more water (Bridge et al., 2013). We investigated whether the changes in WUE that were identified by our screen might arise from differences in rosette architecture between the circadian clock-associated mutants and overexpressors and the wild types.

There was a weak positive correlation between rosette leaf surface area and WUE ($r = 0.400$; $r^2 = 0.160$; $p < 0.001$) (Fig. 3B). Therefore, approximately 16% of variability in WUE can be explained by the variations in rosette leaf surface area that arise from misregulation of the circadian oscillator.

In comparison, rosette leaf surface area was strongly correlated with each of the individual parameters of water used and dry biomass accumulated. The variation in rosette surface area accounted for 83% of the variability in water transpired across the genotypes (Fig. 3C). Furthermore, the variation in rosette surface area accounted for 73% of the variability in biomass accumulation across the genotypes (Fig. 3D), which is unsurprising given that larger leaves are likely to contain more biomass. This demonstrates that one way that circadian regulation affects WUE is through the influence of the circadian oscillator upon
plant development and rosette architecture, but this variation in leaf area does not account for the majority of the influence of circadian regulation upon WUE. It also further supports the notion that the influence of the circadian oscillator upon WUE is complex, and cannot be explained by variation in one of water use or biomass accumulation alone.

Circadian regulation within guard cells alone contributes to water use efficiency

Next, we identified that the circadian oscillator within guard cells contributes to WUE. There is evidence that guard cells contain a circadian oscillator that regulates stomatal opening (Gorton et al., 1989; Hassidim et al., 2017). To investigate the contribution of the guard cell circadian oscillator to WUE, we overexpressed two circadian oscillator components (*CCA1*, *TOC1*) in guard cells, using two guard cell-specific promoters (*GC1*, *MYB60*) for each of *CCA1* and *TOC1* (Fig. 4A) (Cominelli et al., 2005; Galbiati et al., 2008; Yang et al., 2008; Nagy et al., 2009; Meyer et al., 2010; Cominelli et al., 2011; Bauer et al., 2013; Rusconi et al., 2013). *GC1* is a strong guard cell-specific promoter that is relatively unresponsive to a variety of environmental cues (cold, light, ABA, gibberellin) (Yang et al. 2008). We used the full-length *MYB60* promoter sequence, because truncated and chimeric versions of this promoter appear to have weaker activity and/or become rapidly downregulated by dehydration and ABA (Francia et al., 2008; Cominelli et al., 2011; Rusconi et al., 2013). This produced four sets of transgenic lines; *GC1::CCA1:nos (GC), GC1::TOC1:nos (GT), MYB60::CCA1:nos (MC) and MYB60::TOC1:nos (MT)*. We termed these guard cell specific (GCS) plants. We confirmed the guard cell specificity of the *GC1* and *MYB60* promoters in our hands, by driving green fluorescent protein (GFP) under the control of these promoters. GFP accumulation was restricted to the guard cells (Fig. S2A, B). There was not a circadian oscillation in the activity of either the *GC1* or *MYB60* promoter under our experimental conditions (Fig. S2C), demonstrating that these promoters are appropriate for constitutive overexpression of circadian oscillator components within guard cells under our experimental conditions.
To further verify the guard cell-specific overexpression of CCA1 and TOC1 in the GCS plants, we examined CCA1 and TOC1 transcript accumulation within guard cells. Under constant light conditions, we measured CCA1 transcript accumulation in epidermal peels at dusk (when CCA1 transcript abundance is normally low in the wild type) and TOC1 transcript accumulation at dawn (when TOC1 transcript abundance is normally low in the wild type). Guard cell CCA1 overexpressors had greater CCA1 transcript abundance in epidermal peels at dusk than the wild type ($GC$: $t_4 = -2.233, p>0.05$; $MC$: $t_4 = -7.409, p = 0.002$) (Fig. S2D), and guard cell TOC1 overexpressors had greater TOC1 transcript abundance at dawn than the wild type ($GT$: $t_4 = -6.636, p = 0.003$; $MT$: $t_4 = -2.736, p = 0.050$) (Fig. S2D). These data indicate that CCA1 and TOC1 were overexpressed within the guard cells of the guard cell-specific CCA1 or TOC1 overexpressor plants that we generated, respectively.

We investigated the effect on WUE of overexpression of CCA1 and TOC1 within guard cells. Two independent GC1::CCA1 lines (GC-1 and GC-2) were significantly more water use efficient than the wild type ($GC$-1: $p < 0.001$; $GC$-2: $p = 0.002$) (Fig. 4B). $GC$-1 and $GC$-2 were 8% and 4% more water use efficient than the wild type, respectively (Fig. 4B). Two independent MYB60::CCA1 lines also had numerically higher WUE than the wild type, but this was not statistically significant ($p > 0.05$) (Fig. 4B). In contrast, overexpression of TOC1 in guard cells with both the GC1 and MYB60 promoters did not alter WUE ($p > 0.05$) (Fig. 4B). Together, these data suggest that overexpressing CCA1 in guard cells can increase whole plant long-term WUE.

A previous study identified that constitutive overexpression of TOC1 (TOC1-ox) reduces the dehydration tolerance of seedlings (Legnaioli et al., 2009). We wished to determine whether this altered dehydration tolerance is due specifically to the circadian oscillator within guard cells. Using a similar experimental system to Legnaioli et al. 2009, we found that MYB60::CCA1 and GC1::CCA1 increase dehydration survival (Fig. 4C). In contrast, GC1::TOC1 and MYB60::TOC1 had decreased dehydration survival relative to the wild type.
This suggests that overexpressing *CCA1* or *TOC1* in guard cells can increase or decrease survival to dehydration under constant light conditions, respectively.

Like *MYB60::CCA1* and *GC1::CCA1*, more seedlings constitutively overexpressing CCA1 (CCA1-ox) survived dehydration under our experimental conditions (Fig. 4C). Similarly, like *GC1::TOC1* and *MYB60::TOC1*, more seedlings overexpressing TOC1 constitutively (TOC1-ox) were killed by dehydration (Fig. 4C). Therefore, manipulation of the expression of these clock genes in guard cell and whole plants causes similar phenotypes, with some differences in magnitude (Fig. 4C). One interpretation is that altered dehydration survival in CCA1-ox and TOC1-ox seedlings might be partly or wholly due to the circadian clock that is specifically within guard cells. Because the stomatal density was unaltered relative to the wild type in the guard cell overexpressors of CCA1 and TOC1 (Fig. 4D, E), the WUE and dehydration survival phenotypes that we identified might be due to alterations in processes within guard cells rather than due to altered stomatal density.

**Discussion**

_Pervasive influence of the circadian oscillator upon water use efficiency_

Our data indicate that the circadian oscillator is important for regulating the long-term WUE of Arabidopsis. Misregulation of several functional subsections of the circadian oscillator altered the WUE of Arabidopsis. Misexpression of morning (PRR7, PRR9, CCA1), late day (GI, PRR5) and evening (TOC1, ZTL, ELF3) components of the circadian oscillator all perturb WUE under our experimental conditions (Fig. 1, Fig. 2B). Additionally, altered expression of TEJ and GRP7 also alters WUE (Fig. 1). Therefore, oscillator components that impact WUE are not confined to a specific region or expression phase of the multi-loop circadian oscillator. Misexpression of genes encoding some proteins that provide environmental inputs to the circadian oscillator (ELF3, TPS1, ZTL, KIN10; (Covington et al., 2001; Kim et al., 2007; Shin et al., 2017; Frank et al., 2018)) also alters WUE. Together, this suggests that the entire circadian oscillator influences WUE, and that alterations in water use that are caused by mutations to the circadian oscillator are not confined to a specific
sub-loop of the circadian oscillator or restricted to its input or output pathways. One
explanation for these circadian-system wide alterations in WUE relates to the nature of
feedback within the circadian oscillator. The complex feedback and interconnectivity of the
circadian oscillator means that individual components of the circadian oscillator that directly
influence stomatal function or water use are likely to be altered by mutations that are distal
to that component. Therefore, if correct circadian timing is required for optimum water use
efficiency, multiple components of the circadian oscillator are likely to influence water use
efficiency.

The sugar signalling proteins TPS1 and KIN10 influence a broad range of phenotypes, in
addition to participating in circadian entrainment (Baena-González et al., 2007; Gómez et al.,
2010; Paul et al., 2010; Delatte et al., 2011; Shin et al., 2017; Frank et al., 2018; Nietzsche
et al., 2018; Simon et al., 2018). The tps1-12 TILLING mutant of TPS1 decreases stomatal
aperture and increases the ABA sensitivity of guard cells (Gómez et al., 2010), whereas we
found that tps1-11 and tps1-12 had lower long-term WUE than the wild type (Fig. 1).
Reduced biomass accumulation in tps1-11 and tps1-12 (Fig. 2A) was consistent with slow
growth of these alleles (Gómez et al., 2010). Overall, this suggests that the decreased
stomatal aperture of tps1-12 mutants does not translate into an overall increase in WUE,
potentially due to slower growth of the tps1 mutants (Fig. 2A) (Gómez et al., 2010). The
broad range of phenotypes that are altered in tps1-11, tps1-12 and KIN10-ox 6.5 indicates
that these genotypes might alter WUE through mechanisms other than circadian regulation.

Potential roles for the evening complex in WUE

Our finding that ELF3 is important for WUE (Fig. 1) is supported by previous evidence.
Under constant light conditions, wild type Arabidopsis has circadian rhythms of stomatal
aperture, whereas elf3 stomata are constantly open and unresponsive to light and dark
(Kinoshita et al., 2011). Furthermore, ELF3 negatively regulates blue light-mediated stomatal
opening (Kinoshita and Hayashi, 2011). Therefore, perturbation of the anticipation of
day/night transitions or responses to environmental cues in elf3 stomata might cause long-term alterations in WUE.

ELF3 binds to the PRR9 promoter and elf3-1 has elevated PRR9 transcript abundance (Thines and Harmon, 2010; Dixon et al., 2011; Herrero et al., 2012). The low WUE of elf3-1 might potentially be caused by altered PRR9 expression, because misregulation of PRR9 also affected WUE (Fig. 1). In a similar fashion, ELF3/ELF4 signalling represses PRR7, and elf3-1 has elevated PRR7 transcript abundance (Herrero et al., 2012). Under light-dark cycles, elf3-1 also has high and constitutive GI expression (Fowler et al., 1999), and elf3-1 and gi mutants have opposite WUE phenotypes (Fig. 1). Therefore, the WUE phenotype of elf3-1 (Fig. 1) might be caused by disruption of ELF3 itself, or alterations in PRR7, PRR9 and/or GI expression.

Mutating further components of the evening complex (EC) (ELF4 and LUX) did not affect WUE (Fig. 1). This is despite the way that these genes influence circadian oscillator function and plant physiology (Hsu and Harmer, 2014; Huang and Nusinow, 2016), and nocturnal regulation of stomatal aperture impacts WUE (Costa et al., 2015; Coupel-Ledru et al., 2016). One possibility is that the impact of elf3 on WUE may be greater than that of elf4 or lux because ELF3 is key to EC scaffolding, with ELF3 operating genetically downstream from ELF4 and LUX (Herrero et al., 2012; Huang and Nusinow, 2016).

ELF4 appears to play a greater role in circadian regulation in the vascular tissue than stomatal guard cells, with vasculature expression up to ten times higher than other tissues (Endo et al., 2014). Because elf3-1 affects WUE differently from elf4-101 and lux-1 (Fig. 1), it appears that ELF3 regulates WUE independently from ELF4 and LUX.

Multiple physiological causes of altered WUE in circadian oscillator mutants

Our data suggest that changes in WUE caused by misexpression of circadian clock components might be due to a combination of physiological factors. Some mutants or overexpressors tested alter biomass accumulation, whilst others predominantly alter water
loss (Fig. 2), so mutations to the circadian oscillator did not alter water use by specifically altering one of carbon assimilation or transpiration. This is consistent with previous work demonstrating that both stomatal opening and CO₂ fixation is perturbed in circadian arrhythmic plants under light/dark cycles (Dodd et al., 2005), and with the findings that daily carbohydrate management is dependent upon correct circadian regulation (Graf et al., 2010). We speculate that delayed or advanced stomatal and photosynthetic responses to the day-night cycle might occur in circadian period mutants, because period mutants inaccurately anticipate the onset of dawn (Dodd et al., 2014). Circadian clock mutants might also affect WUE by changing the sensitivity of stomatal movements and photosynthesis to environmental transitions, because there is circadian gating of the responses of both stomata and photosynthesis to environmental cues (Dodd et al., 2006; Kinoshita et al., 2011; Litthauer et al., 2015; Joo et al., 2017; Cano-Ramirez et al., 2018). Some effects of the circadian oscillator upon WUE arise from alterations in leaf size that occur in some circadian oscillator mutants (Fig. 3A, B). This suggests that developmental alterations arising from lesions in the circadian oscillator can lead to changes in WUE. Such developmental alterations might alter WUE by changing airflow around the rosette, boundary layer conductance, or internal leaf structure.

Conclusions

We show that circadian regulation contributes to whole plant long-term WUE under cycles of day and night. This control occurs partly through the influence of the circadian oscillator upon rosette architecture. Mutation or overexpression of CCA1, TOC1, ELF3, GI, GRP7, PRR5, PRR7, PRR9, TEJ and ZTL altered WUE under our experimental conditions. The roles of these genes in WUE may be independent or overlapping, and their WUE phenotypes might be due to direct effects of these genes, or indirect effects on transcript and/or protein abundance of other circadian clock gene(s). Misregulation of the expression of CHE, FKF1, LKP2, RVE4, RVE8, PRR3, ELF4, LUX and WNK1 did not appear to alter WUE under our experimental conditions.
Our results have a number of broad implications. Firstly, our data suggest that alterations in circadian function that arise during crop breeding could have the potential to increase or decrease WUE. Therefore, manipulation of the functioning of the circadian oscillator might represent a pathway to tune the WUE of crops. Second, our results indicate that circadian regulation in a single cell type can have implications for whole-plant physiology. Finally, our findings suggest that circadian regulation can alter a single trait (WUE) by affecting many aspects of physiology. In future, it would be informative to distinguish the contribution to overall WUE of circadian regulation within additional cell types, such as the mesophyll, vascular tissue, and root cell types.

Materials and methods

Plant material and growth conditions

Arabidopsis (Arabidopsis thaliana (L.) Heynh.) seeds were surface-sterilised as described previously (Noordally et al., 2013). For experiments investigating stomatal density and index, seeds were stratified for 3 days at 4 °C, then sown on compost mix comprising a 3:1 ratio of coarsely sieved Levington Advance F2 seed compost (Everris) and horticultural silver sand (Melcourt), supplemented with 0.4 g l⁻¹ thiacloprid insecticide granules (Exemptor; Everris). Plants were grown in controlled environment chambers (Reftech, Netherlands) under an 8 h photoperiod at 70% humidity, 20 °C, and photon flux density of 100 µmol m⁻² s⁻¹ of overhead lighting supplied by cool white fluorescent tubes (Reftech, Netherlands). For experiments investigating long-term WUE, seeds were sown within a custom Falcon tube system then stratified. Plants were cultivated in plant growth chambers (Snijder, Netherlands) under the experimental conditions described above. The genotypes that were screened for WUE alterations are identified in Table S1, and all have been described previously. For all experiments, at least two completely independent experimental repeats were performed per genotype and per treatment, with multiple replicate plants within each of the experimental repeats.
Generation of transgenic lines

To create the \textit{GC1::CCA1:nos} (GC), \textit{GC1::TOC1:nos} (GT), \textit{MYB60::CCA1:nos} (MC) and \textit{MYB60::TOC1:nos} (MT) constructs, the CaMV \textit{nos} terminator sequence was ligated between the SpeI and NotI restriction sites in the pGREENII0229 binary vector (Hellens et al., 2000). The \textit{GC1} upstream sequence (-1894 to -190) or \textit{MYB60} upstream sequence (-1724 to -429) was then ligated between the KpnI and ApaI restriction sites of pGREENII0229. Finally, the \textit{CCA1} coding sequence or \textit{TOC1} coding sequence, obtained using RT-PCR, was ligated between the restriction Xhol and Xmal sites. Primers used are identified in Table S2. Constructs were transformed into Col-0 wild type Arabidopsis using transformation with \textit{Agrobacterium tumefaciens} strain GV3101. Transformants were identified by screening for phosphinothricin resistance, then further validated using genomic DNA PCR. Homozygous lines were identified via phosphinothricin (BASTA) resistance, and two independently transformed homozygous lines were investigated in detail per genotype.

Guard cell specificity of promoter activity was investigated using \textit{GC1::GFP:nos} and \textit{MYB60::GFP:nos} promoter-reporter lines (Sup. Fig. 3A-C), which were created as above with the \textit{GFP} coding sequence ligated between the Xhol and Xmal restriction sites. Leaf discs (5 mm diameter) from seedlings or mature plants were mounted on microscope slides with dH$_2$O, and examined for GFP fluorescence using confocal microscopy (Leica DMI6000).

The following settings were used: argon laser at 20\% capacity, 488 nm laser at 48\% capacity with a bandwidth of 505 nm–515 nm, gain of 1250, offset at 0.2\%, 20x or 40x objective, zoom x1 to x4.

Measurement of water use efficiency

The WUE assay was adapted from Wituszynska et al. (2013) (Wituszyńska et al., 2013). Plants were grown for 6 weeks in modified 50 ml Falcon tubes. The Falcon tube systems consisted of a 50 ml Falcon tube filled with 37.5 ml of a 1:1 ratio of compost: perlite and 35 ml of Milli-Q water (Merck), with the remaining volume filled with a 1:1 ratio of compost: Milli-Q water (Fig. S3). Each Falcon tube lid had a 2 mm diameter hole drilled in its centre to
allow plant growth. The lid was spray-painted black (Hycote) because we found that the orange colour of the Falcon tube lid caused leaf curling (Fig. S3). The system was wrapped in aluminium foil to exclude light (Fig. S3). 10-15 seeds were sown through the Falcon tube lid using a pipette. Following stratification, Falcon tube systems were placed under growth conditions using a randomised experimental design. 7 days after germination, seedlings were trimmed to one per Falcon tube system, and initial Falcon tube weight was recorded. After 6 weeks of growth, rosette leaf surface area was measured by photography (D50; Nikon) and Fiji software, rosette dry weight was measured (4 d at 60°C), and final Falcon tube weight was recorded. Negative controls (Falcon tube systems without plants) were used to assess soil water evaporation over 18 experimental repeats, with an overall mean weight loss of 0.513 g ± 0.004 g over 6 weeks for plant-free Falcon tubes.

Plant WUE was calculated as follows:

$$WUE = \frac{d}{(t_i - t_f) - e}$$

Where $d$ is the rosette dry weight at the end of the experiment (mg), $t_i$ and $t_f$ are the falcon tube weight at the start and end of the experiment, respectively (g), and $e$ is the amount of water evaporation directly from the compost (g). WUE is derived as mg biomass per ml water lost. These calculations assumed that 1 g of weight change was equivalent to a change of 1 ml of water. For each of 3 independent experimental repeats, 15 plants were screened per genotype. Due to variation between the WUE of each background (Fig. S1), the WUE of each circadian oscillator genotype was normalized to its respective background and expressed as a percentage of that background. Statistical comparisons with the wild types were conducted before this normalization.

**Dehydration tolerance assay**

This assay was adapted from Legnaioli et al. (2009). For experiments investigating survival to dehydration, surface-sterilized seeds were sown on Petri dishes containing half strength Murashige & Skoog basal salt mixture (0.5x MS) (Duchefa), supplemented with 0.8% (w/v)
agar and 3% (w/v) sucrose, then stratified for 3 days at 4 °C before transfer to the growth chamber. For these experiments, seedlings were cultivated in MLR-352 growth chambers (Panasonic) at 19°C with photon flux density of 120 µmol m⁻² s⁻¹. 14-day old seedlings were dehydrated on a double layer of filter paper (Fisher Scientific) for 9 h under constant light conditions, watered with sterile dH₂O, and kept under constant light conditions for a further 48 h before being scored for survival. Seedlings with a green apical meristematic region were counted as survivors. 32 seedlings were treated per genotype and within each experimental repeat.

Measurement of stomatal density

Plants were grown for 7-8 weeks on compost mix. Dental paste (Coltene) was applied to the abaxial surface of fully expanded leaves. Transparent nail varnish (Rimmel) was applied to these leaf moulds once they had set, and then peeled away from the mould using clear adhesive tape (Scotch Crystal). Stomatal and pavement cells were counted within an 800 µm x 800 µm square at the centre of each leaf half, using an epifluorescence microscope (HAL100; Zeiss) and Volocity (Perkin Elmer) and Fiji software. For each experimental repeat, two leaves were sampled per plant and eight plants sampled per genotype. Stomatal index was calculated as follows:

\[ SI = \frac{s}{s + p} \times 100 \]

Where SI is the stomatal index, s the number of stomata in the field of view (800 µm x 800 µm), and p the number of pavement cells in the field of view.

RNA extraction and qRT-PCR

RNA extractions, cDNA synthesis, and qRT-PCR were performed according to (Simon et al., 2018), except approximately 10 seedlings were used per RNA sample and analysis was performed using an MXPro 3005 real time PCR system (Agilent) with 5x HOT FIREPol EvaGreen qPCR mastermix (Solis Biodyne). qRT-PCR primers are provided in Table S3. Rhythmic features within qPCR data were identified using the BioDare2 platform (Zielinski et
al., 2014), using the Fast Fourier Transform Non-Linear Least Squares method (FFT-NLLS).

One independently-transformed line of each guard cell-specific circadian clock gene overexpressor was also investigated using qRT-PCR conducted on RNA isolated from epidermal peels. Abaxial leaf epidermis was detached, then washed in 10 mM MES (pH 6.15, adjusted using 10 M KOH) to remove RNA derived from ruptured epidermal cells. Each RNA sample was derived from 20 epidermal peels (five plants, four leaves per plant) that were collated and flash-frozen in liquid nitrogen. Guard cell RNA was extracted using the RNeasy UCP Micro Kit (Qiagen) according to manufacturer’s instructions, with the following modification: guard cell lysis was performed by adding glass beads (425 μm - 600 μm diameter, acid washed, from Sigma-Aldrich) and 350 μl RULT buffer to the sample, then vortexed for 5 min.

Accession numbers

Arabidopsis Genome Initiative identifiers for the genes mentioned in this study are: CCA1 (CIRCADIAN CLOCK ASSOCIATED1, At2g46830), CHE (CCA1 HIKING EXPEDITION, At5g08330), ELF3 (EARLY FLOWERING3, At2g25930), ELF4 (EARLY FLOWERING4, At2g40080), FKF1 (F BOX1, At1g68050), GI (GIGANTEA, At1g22770), GRP7 (GLYCINE RICH PROTEIN7, At2g21660), KIN10 (SNF1-RELATED PROTEIN KINASE1.1, At3g01090), LKP2 (LOV KELCH PROTEIN2, At2g18915), LUX (LUX ARRHYTHMO, At3g46640), MYB60 (MYB DOMAIN PROTEIN60, At1g08810), PRR3 (PSEUDO-RESPONSE REGULATOR3, At5g60100), PRR5 (PSEUDO-RESPONSE REGULATOR5, At5g24470), PRR7 (PSEUDO-RESPONSE REGULATOR7, At5g02810), PRR9 (PSEUDO-RESPONSE REGULATOR9, At2g46790), RVE4 (REVEILLE4, At5g02840), TEJ (POLY(ADP-RIBOSE)GLYCOHYDROLASE1, At2g31870), TIC (TIME FOR COFFEE, At3gt22380), TOC1 (TIMING OF CAB EXPRESSION1, At5g61380), TPS1 (TREHALOSE-6-PHOSPHATE SYNTHASE1, At1g78580), WNK1 (WITH NO LYSINE KINASE1, At3g04910), ZTL (ZEITLUPE, At5g57360).
Acknowledgements

We thank Kester Cragg-Barber, James Chen, Ioanna Kostaki, Jean-Charles Isner, Deirdre McLachlan, Peng Sun, Ashutosh Sharma and Dora Cano-Ramirez for technical advice during experimentation. We thank Marc Knight and Tracy Lawson for helpful discussions concerning data interpretation. We are grateful to Keara Franklin, Alex Webb, Paloma Mas, Steve Kay, Isabelle Carre, Takato Imaizumi, Filip Rolland, Ian Graham, Stacey Harmer, and Steven Penfield for donating seed lines for this study. This research was funded the UK Biotechnology and Biological Sciences Research Council (BBSRC; grant BB/J014400/1) and the Wolfson Foundation.
Figure legends

**Figure 1.** The circadian clock regulates long-term water use efficiency of Arabidopsis under light/dark cycles. The WUE of circadian clock mutants and overexpressors is expressed as a percentage of their respective background (normalized to 100%, red reference line) to account for WUE variation between background accessions \( n = 5 - 15 \). Data were analysed using independent-samples t-tests and statistical significance is indicated relative to the background using \( * = p < 0.05; ** = p < 0.01; *** = p < 0.001 \). Statistical analysis was performed on raw data, with data subsequently converted to a percentage of the wild type for the purposes of comparison and presentation. Screens were repeated independently three times per genotype, with one representative experimental repeat shown here for each genotype.

**Figure 2.** Altered WUE of plants with mutations or overexpression of circadian clock associated genes is not caused consistently by variation in one of dry weight, water use, phase of expression of each gene, or resultant altered period or flowering time. Data are derived from Fig. 1 and expressed as a percentage of the respective background (WT, normalised to 100%, red reference line) \( n = 5 - 15 \). (A) Altered WUE is not specifically due to altered water use or altered dry weight of screened genotypes, but results from the combination of both. (b-d) Variations in WUE are not explained by (B) phase expression of mutated/overexpressed gene, nor by altered (C) period or (D) flowering time of the mutated/overexpressor genotype. Genotypes reported to have no change (N/C) in period or flowering time relative to the wild type are included on the left of panels (B) and (C), while those for which period and/or flowering time are unknown are included on the right. Studies describing the phase of expression, period and flowering time of the genotypes tested are identified in the main text. We note that the phase of expression and period data used for this analysis were often obtained under constant conditions, in contrast to our experiments occurring under light/dark cycles.
**Figure 3.** The circadian oscillator alters WUE partially by changing rosette architecture. (A)

Altering circadian-associated gene expression can affect rosette architecture and size, as illustrated for *elf3-1*, *lux-1*, and *gi-2* in (Col-0 background). Image backgrounds removed for clarity. Variation in rosette leaf surface area across the genotypes investigated explained (B) 16% of variation in WUE (*p* < 0.001, *r* = 0.400, *r*² = 0.160), (C) 83% of variation in transpiration (*p* < 0.001, *r* = 0.912, *r*² = 0.832) and (D) 73% of variation in rosette dry biomass (*p* < 0.001, *r* = 0.857, *r*² = 0.734). Data were analysed using Pearson correlation tests.

**Figure 4.** Overexpressing *CCA1* or *TOC1* in guard cells affects WUE and survival of dehydration by seedlings. (A) Constructs used to overexpress *CCA1* or *TOC1* coding sequence under control of *GC1* or *MYB60* promoters. (B) Guard cell *CCA1* overexpression can increase WUE. WUE expressed as a percentage of the wild type (normalised to 100%, red reference line). Two to four independent experimental repeats were performed, with data from one representative dataset shown (*n* = 5 - 15). Data for *CCA1*-ox and *TOC1*-ox are derived from Fig. 1, for purposes of comparison. Data were analysed with independent samples t-tests, and statistical significance compared to Col-0 is indicated using starring (** = *p* < 0.01; *** = *p* < 0.001). (C) Guard cell *CCA1* or *TOC1* overexpression alters dehydration survival of seedlings compared with the wild type. Data were obtained from three independent experimental repeats (mean; *n* = 32 per experimental replicate; at least two independent experimental repeats were performed for each genotype). A single *GC1::TOC1* line is shown here because other lines produced extremely variable data. (D,E) Guard cell *CCA1* or *TOC1* overexpression does not affect (D) stomatal index nor (E) stomatal density. Two independent experimental repeats were performed, with data from one representative dataset shown (*n* = 19 - 32; mean ± S.E.M.). Data were analysed with ANOVA and Tukey’s post hoc tests (NS = *p* > 0.05). Bar colours identify the whole plant overexpressor control (black), wild type control (dark grey), and guard cell-specific overexpressor genotypes (light grey).
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**Figure 1.** The circadian clock regulates long-term water use efficiency of Arabidopsis under light/dark cycles. The WUE of circadian clock mutants and overexpressors is expressed as a percentage of their respective background (normalized to 100%, red reference line) to account for WUE variation between background accessions \((n = 5 - 15)\). Data were analysed using independent-samples t-tests and statistical significance is indicated relative to the background using \(* = p < 0.05; ** = p < 0.01; *** = p < 0.001\). Statistical analysis was performed on raw data, with data subsequently converted to a percentage of the wild type for the purposes of comparison and presentation. Screens were repeated independently three times per genotype, with one representative experimental repeat shown here for each genotype.
Figure 2. Altered WUE of plants with mutations or overexpression of circadian clock associated genes is not caused consistently by variation in one of dry weight, water use, phase of expression of each gene, or resultant altered period or flowering time. Data are derived from Fig. 1 and expressed as a percentage of the respective background (WT, normalised to 100%, red reference line) \((n = 5 - 15)\). (A) Altered WUE is not specifically due to altered water use or altered dry weight of screened genotypes, but results from the combination of both. (b-d) Variations in WUE are not explained by (B) phase expression of mutated/overexpressed gene, nor by altered (C) period or (D) flowering time of the mutated/overexpressor genotype. Genotypes reported to have no change (N/C) in period or flowering time relative to the wild type are included on the left of panels (B) and (C), while
those for which period and/or flowering time are unknown are included on the right. Studies describing the phase of expression, period and flowering time of the genotypes tested are identified in the main text. We note that the phase of expression and period data used for this analysis were often obtained under constant conditions, in contrast to our experiments occurring under light/dark cycles.
Figure 3. The circadian oscillator alters WUE partially by changing rosette architecture. (A) Altering circadian-associated gene expression can affect rosette architecture and size, as illustrated for elf3-1, lux-1, and gi-2 in (Col-0 background). Image backgrounds removed for clarity. Variation in rosette leaf surface area across the genotypes investigated explained (B) 16% of variation in WUE ($p < 0.001$, $r = 0.400$, $r^2 = 0.160$), (C) 83% of variation in transpiration ($p < 0.001$, $r = 0.912$, $r^2 = 0.832$) and (D) 73% of variation in rosette dry biomass ($p < 0.001$, $r = 0.857$, $r^2 = 0.734$). Data were analysed using Pearson correlation tests.
Figure 4. Overexpressing CCA1 or TOC1 in guard cells affects WUE and survival of dehydration by seedlings. (A) Constructs used to overexpress CCA1 or TOC1 coding sequence under control of GC1 or MYB60 promoters. (B) Guard cell CCA1 overexpression can increase WUE. WUE expressed as a percentage of the wild type (normalised to 100%, red reference line). Two to four independent experimental repeats were performed, with data from one representative dataset shown (n = 5 - 15). Data for CCA1-ox and TOC1-ox are derived from Fig. 1, for purposes of comparison. Data were analysed with independent samples t-tests, and statistical significance compared to Col-0 is indicated using starring (** = p < 0.01; *** = p < 0.001). (C) Guard cell CCA1 or TOC1 overexpression alters dehydration survival of seedlings compared with the wild type. Data were obtained from three independent experimental repeats (mean; n = 32 per experimental replicate; at least
two independent experimental repeats were performed for each genotype). A single GC1::TOC1 line is shown here because other lines produced extremely variable data. (D, E) Guard cell CCA1 or TOC1 overexpression does not affect (D) stomatal index nor (E) stomatal density. Two independent experimental repeats were performed, with data from one representative dataset shown ($n = 19 - 32$; mean ± S.E.M.). Data were analysed with ANOVA and Tukey’s post hoc tests (NS = $p > 0.05$). Bar colours identify the whole plant overexpressor control (black), wild type control (dark grey), and guard cell-specific overexpressor genotypes (light grey).
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