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.

49 Plant water loss can be manipulated through changes in the regulation of stomatal opening
50 and by altering stomatal density and patterning (Pei et al., 1998; Hugouvieux et al., 2001;
51 Schroeder et al., 2001; Hetherington and Woodward, 2003; Yoo et al., 2010; Lawson and
52 Blatt, 2014; Franks et al., 2015; Caine et al., 2019). In addition to stomatal responses to
53 environmental cues such as light, temperature and phytohormones, there are circadian
54 rhythms of stomatal opening (Gorton et al., 1989; Hennessey and Field, 1991). Circadian
55 rhythms are self-sustaining biological cycles with a period of about 24 h. These rhythms are
56 thought to adapt plants to daily cycles of light and dark, by anticipating daily changes in the
57 environment and co-ordinating cellular processes. In higher plants, circadian rhythms are
58 generated by several interlocked transcription-translation feedback loops known as the
59 circadian oscillator (Hsu and Harmer, 2014). The phase of the circadian oscillator is adjusted
60 continuously to match the phase of the environment through the process of entrainment, in
61 response to light, temperature and metabolic cues (Somers et al., 1998; Millar, 2004;
62 Salomé and McClung, 2005; Haydon et al., 2013). Additionally, the circadian oscillator
63 communicates an estimate of the time of day to circadian-regulated features of the cell,
64 initially through transcriptional regulation (Harmer et al., 2000). The known circadian
65 oscillator controls circadian rhythms of stomatal opening because mutations that alter the
66 circadian period or cause circadian arrhythmia lead to equivalent alterations in the circadian
67 rhythm of stomatal opening (Somers et al., 1998; Dodd et al., 2004; Dodd et al., 2005). The
68 circadian oscillator is also involved in the responses of guard cells to environmental cues
69 such as drought and low temperature (Dodd et al., 2006; Legnaioli et al., 2009).

70 Circadian rhythms are often studied under conditions of constant light. However, the
71 circadian oscillator is also important for the regulation of stomatal opening under cycles of
72 light and dark. For example, overexpression of the circadian oscillator component CCA1
73 (CCA1-ox) alters the daily regulation of stomatal opening such that stomatal conductance
74 increases steadily throughout the photoperiod (Dodd et al., 2005). In comparison, in wild
75 type plants stomatal conductance remains relatively uniform during the photoperiod and is

76 substantially lower than CCA1-ox (Dodd et al., 2005). This suggests that misregulation of the
77 circadian oscillator might alter plant water use under cycles of light and dark.
78 Overexpression of CCA1 specifically within guard cells, using a guard cell specific promoter,
79 alters flowering time and drought response phenotypes under cycles of light and dark
80 (Hassidim et al., 2017). Like constitutive CCA1 overexpression (Dodd et al., 2005), CCA1
81 overexpression specifically within guard cells generally causes greater stomatal opening
82 during the light period (Hassidim et al., 2017). Therefore, the circadian oscillator within guard
83 cells is important for the daily regulation of stomatal opening (Hassidim et al., 2017).
84 Modelling suggests that under light/dark cycles, the circadian oscillator contributes at the
85 canopy scale to daily rhythms in stomatal aperture and carbon assimilation in bean and
86 cotton (Resco de Dios et al., 2016).

87 The contribution of the circadian oscillator to both stomatal opening and growth and biomass
88 accumulation (Dodd et al., 2005; Graf et al., 2010) suggests that the circadian oscillator
89 might make an important contribution to water use efficiency (WUE). WUE is the ratio of
90 carbon dioxide incorporated through photosynthesis into biomass to the amount of water lost
91 through transpiration. At the single leaf level, instantaneous, intrinsic WUE is often
92 measured with gas exchange techniques and expressed as net CO_2 assimilation per unit of
93 water transpired (Vialet-Chabrand et al., 2016; Ruggiero et al., 2017; Ferguson et al., 2018).
94 However, such measurements do not provide an accurate representation of WUE over the
95 plant lifetime, which is influenced by features such as leaf position, dark respiration, and time
96 of day changes in instantaneous WUE (Condon et al., 2004; Tomás et al., 2014; Medrano et
97 al., 2015; Ferguson et al., 2018). It is important to note that WUE is not a drought resistance
98 trait (Blum, 2009).

99 Given that the circadian oscillator affects stomatal opening and biomass accumulation
100 (Gorton et al., 1989; Hennessey and Field, 1991; Dodd et al., 2005; Edwards and Weinig,
101 2010; Graf et al., 2010; Edwards et al., 2012), we hypothesized that specific components of
102 the circadian oscillator might make an important contribution to long-term WUE of plants.

103 Therefore, we investigated the impact of the misregulation of parts of the circadian oscillator
104 upon the long-term WUE of Arabidopsis. We identified that the circadian oscillator has
105 profound effects upon the long-term WUE of plants. Importantly, some alterations in
106 oscillator function increase long-term WUE, suggesting potential targets for future
107 improvements of crop WUE.

108 **Results**

109 *Circadian oscillator components contribute to water use efficiency*

110 Each background accession had a distinct WUE (C24: 3.01 ± 0.07 mg ml^{-1}; Col-0: 2.22 ±
111 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
112 differences between backgrounds are consistent with previous studies of WUE, stomatal
113 function and stomatal density in Arabidopsis (Nienhuis et al., 1994; Woodward et al., 2002;
114 Dodd et al., 2004; Masle et al., 2005; Karaba et al., 2007; Ruggiero et al., 2017; Ferguson et
115 al., 2018).

116 We identified that correct regulation of the circadian oscillator makes a substantial
117 contribution to WUE. 33 single mutants or overexpressors of genes associated with
118 circadian regulation, representing 22 circadian oscillator-associated components, were
119 screened for WUE (Fig. 1). Nearly half of the mutants or overexpressors examined had a
120 significantly different WUE from the wild type ($p < 0.05$; 16 of 33 genotypes). This
121 corresponded to mutants or overexpressors representing half of the circadian oscillator
122 components covered by our study (11 of 22 genes) (Fig. 1). The *cca1*-11, *elf3*-1, *prr5*-3,
123 *prr9*-1, *tps1*-11, *tps1*-12, and *ztl*-1 mutants, as well as the TOC1 and KIN10 (line 6.5)
124 overexpressors, had significantly lower WUE than the wild type (Fig. 1). The *gi*-2, *gi*-11,
125 *grp7*-1, *prr7*-11 and *tej*-1 mutants had significantly greater WUE than the wild type (Fig. 1).
126 This suggests that misregulating the expression of circadian clock components CCA1, ELF3,
127 GI, GRP7, PRR5, PRR7, PRR9, TEJ, TOC1 and ZTL changes whole plant long-term WUE
128 (Fig. 1). *tic*-2 had significantly greater WUE than the wild type, whereas *tic*-1 had
129 significantly lower WUE than the wild type, so the effect of *TIC* mutation upon WUE is

130 unclear (Fig. 1). We also included the *che*-2 mutant in our initial analysis, but inconsistency
131 of its WUE phenotype between experimental repeats led us to exclude the data. WUE was
132 also altered by changing the expression of the energy signalling components TPS1 and
133 KIN10 that participate in inputs to the circadian oscillator (Shin et al., 2017; Frank et al.,
134 2018) (Fig. 1). Therefore, correct expression of these circadian clock-associated genes
135 contributes to long-term WUE of Arabidopsis.

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

142 We hypothesised that variations in WUE might be explained by specific circadian
143 phenotypes in the mutants and overexpressors that we tested. For example, mutations in
144 clock genes expressed with a particular set of phases might have a pronounced effect on
145 WUE. Likewise, the nature of the circadian period change or flowering time change resulting
146 from misexpression of each oscillator component might be associated with certain changes
147 in WUE. To test this, we related the data from our WUE screen to the circadian phase of
148 expression of each mutated or overexpressed gene. We also compared the direction of
149 change of WUE to the period and flowering time phenotypes that arise from each mutant or
150 overexpressor (Fowler et al., 1999; Schultz et al., 2001; Doyle et al., 2002; Nakamichi et al.,
151 2002; Yanovsky and Kay, 2002; Imaizumi et al., 2003; Más et al., 2003; Murakami et al.,
152 2004; Farré et al., 2005; Hazen et al., 2005; Baena-González et al., 2007; Streitner et al.,
153 2008; Wang et al., 2008; Baudry et al., 2010; Nakamichi et al., 2010; Rawat et al., 2011;
154 Wahl et al., 2013; Hsu and Harmer, 2014). We note that the phenotypes reported by these
155 studies were often identified under constant conditions, whereas our experiments occurred
156 under light/dark cycles.

157 There was no obvious relationship between the circadian phenotypes that are caused by
158 each mutant or overexpressor investigated and the WUE of each of these lines (Fig. 2B, C,
159 D). For example, mutating morning-phased circadian oscillator components can either
160 decrease or increase WUE (Fig. 2B). Mutants that cause long circadian periods and short
161 circadian periods can both increase and decrease WUE (Fig. 2C). Furthermore, mutants and
162 overexpressors that cause both early and delayed flowering can each increase and
163 decrease WUE (Fig. 2D).

164 *Circadian regulation of water use efficiency combines multiple traits*

165 Mutation or overexpression of components of the circadian oscillator can cause changes in
166 the development of Arabidopsis, such as alterations in rosette size, leaf shape and petiole
167 length (Fig. 3A) (Zagotta et al., 1992; Schaffer et al., 1998; Wang and Tobin, 1998; Dodd et
168 al., 2005; Ruts et al., 2012; Rubin et al., 2018). These changes are likely to have
169 implications for gas exchange because, for example, spatially separated leaves are
170 predicted to transpire more water (Bridge et al., 2013). We investigated whether the changes
171 in WUE that were identified by our screen might arise from differences in rosette architecture
172 between the circadian clock-associated mutants and overexpressors and the wild types.
173 There was a weak positive correlation between rosette leaf surface area and WUE (r =
174 0.400; r^2 = 0.160; p < 0.001) (Fig. 3B). Therefore, approximately 16% of variability in WUE
175 can be explained by the variations in rosette leaf surface area that arise from misregulation
176 of the circadian oscillator.

177 In comparison, rosette leaf surface area was strongly correlated with each of the individual
178 parameters of water used and dry biomass accumulated. The variation in rosette surface
179 area accounted for 83% of the variability in water transpired across the genotypes (Fig. 3C).
180 Furthermore, the variation in rosette surface area accounted for 73% of the variability in
181 biomass accumulation across the genotypes (Fig. 3D), which is unsurprising given that
182 larger leaves are likely to contain more biomass. This demonstrates that one way that
183 circadian regulation affects WUE is through the influence of the circadian oscillator upon

184 plant development and rosette architecture, but this variation in leaf area does not account
185 for the majority of the influence of circadian regulation upon WUE. It also further supports
186 the notion that the influence of the circadian oscillator upon WUE is complex, and cannot be
187 explained by variation in one of water use or biomass accumulation alone.

188 *Circadian regulation within guard cells alone contributes to water use efficiency*

189 Next, we identified that the circadian oscillator within guard cells contributes to WUE. There
190 is evidence that guard cells contain a circadian oscillator that regulates stomatal opening
191 (Gorton et al., 1989; Hassidim et al., 2017). To investigate the contribution of the guard cell
192 circadian oscillator to WUE, we overexpressed two circadian oscillator components (*CCA1*,
193 *TOC1*) in guard cells, using two guard cell-specific promoters (*GC1*, *MYB60*) for each of
194 *CCA1* and *TOC1* (Fig. 4A) (Cominelli et al., 2005; Galbiati et al., 2008; Yang et al., 2008;
195 Nagy et al., 2009; Meyer et al., 2010; Cominelli et al., 2011; Bauer et al., 2013; Rusconi et
196 al., 2013). *GC1* is a strong guard cell-specific promoter that is relatively unresponsive to a
197 variety of environmental cues (cold, light, ABA, gibberellin) (Yang et al. 2008). We used the
198 full-length *MYB60* promoter sequence, because truncated and chimeric versions of this
199 promoter appear to have weaker activity and/or become rapidly downregulated by
200 dehydration and ABA (Francia et al., 2008; Cominelli et al., 2011; Rusconi et al., 2013). This
201 produced four sets of transgenic lines; *GC1::CCA1:nos* (*GC*), *GC1::TOC1:nos* (*GT*),
202 *MYB60::CCA1:nos* (*MC*) and *MYB60::TOC1:nos* (*MT*). We termed these guard cell specific
203 (GCS) plants. We confirmed the guard cell specificity of the *GC1* and *MYB60* promoters in
204 our hands, by driving green fluorescent protein (GFP) under the control of these promoters.
205 GFP accumulation was restricted to the guard cells (Fig. S2A, B). There was not a circadian
206 oscillation in the activity of either the *GC1* or *MYB60* promoter under our experimental
207 conditions (Fig. S2C), demonstrating that these promoters are appropriate for constitutive
208 overexpression of circadian oscillator components within guard cells under our experimental
209 conditions.

210 To further verify the guard cell-specific overexpression of *CCA1* and *TOC1* in the GCS
211 plants, we examined *CCA1* and *TOC1* transcript accumulation within guard cells. Under
212 constant light conditions, we measured *CCA1* transcript accumulation in epidermal peels at
213 dusk (when *CCA1* transcript abundance is normally low in the wild type) and *TOC1*
214 transcript accumulation at dawn (when *TOC1* transcript abundance is normally low in the
215 wild type). Guard cell *CCA1* overexpressors had greater *CCA1* transcript abundance in
216 epidermal peels at dusk than the wild type (*GC*: t_4 = - 2.233, p>0.05; *MC*: t_4 = -7.409, p =
217 0.002) (Fig. S2D), and guard cell *TOC1* overexpressors had greater *TOC1* transcript
218 abundance at dawn than the wild type (*GT*: t_4 = -6.636, p = 0.003; *MT*: t_4 = -2.736, p =
219 0.050) (Fig. S2D). These data indicate that *CCA1* and *TOC1* were overexpressed within the
220 guard cells of the guard cell-specific *CCA1* or *TOC1* overexpressor plants that we
221 generated, respectively.

222 We investigated the effect on WUE of overexpression of CCA1 and TOC1 within guard cells.
223 Two independent *GC1::CCA1* lines (*GC*-1 and *GC*-2) were significantly more water use
224 efficient than the wild type (*GC*-1: p < 0.001; *GC*-2: p = 0.002) (Fig. 4B). *GC*-1 and *GC*-2
225 were 8% and 4% more water use efficient than the wild type, respectively (Fig. 4B). Two
226 independent *MYB60::CCA1* lines also had numerically higher WUE than the wild type, but
227 this was not statistically significant (p > 0.05) (Fig. 4B). In contrast, overexpression of *TOC1*
228 in guard cells with both the *GC1* and *MYB60* promoters did not alter WUE (p > 0.05) (Fig.
229 4B). Together, these data suggest that overexpressing CCA1 in guard cells can increase
230 whole plant long-term WUE.

231 A previous study identified that constitutive overexpression of TOC1 (TOC1-ox) reduces the
232 dehydration tolerance of seedlings (Legnaioli et al., 2009). We wished to determine whether
233 this altered dehydration tolerance is due specifically to the circadian oscillator within guard
234 cells. Using a similar experimental system to Legnaioli et al. 2009, we found that
235 *MYB60::CCA1* and *GC1::CCA1* increase dehydration survival (Fig. 4C). In contrast,
236 *GC1::TOC1* and *MYB60::TOC1* had decreased dehydration survival relative to the wild type

237 (Fig. 4C). This suggests that overexpressing *CCA1* or *TOC1* in guard cells can increase or
238 decrease survival to dehydration under constant light conditions, respectively.

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

250 **Discussion**

251 *Pervasive influence of the circadian oscillator upon water use efficiency*

252 Our data indicate that the circadian oscillator is important for regulating the long-term WUE
253 of Arabidopsis. Misregulation of several functional subsections of the circadian oscillator
254 altered the WUE of Arabidopsis. Misexpression of morning (PRR7, PRR9, CCA1), late day
255 (GI, PRR5) and evening (TOC1, ZTL, ELF3) components of the circadian oscillator all
256 perturb WUE under our experimental conditions (Fig. 1, Fig. 2B). Additionally, altered
257 expression of TEJ and GRP7 also alters WUE (Fig. 1). Therefore, oscillator components that
258 impact WUE are not confined to a specific region or expression phase of the multi-loop
259 circadian oscillator. Misexpression of genes encoding some proteins that provide
260 environmental inputs to the circadian oscillator (ELF3, TPS1, ZTL, KIN10; (Covington et al.,
261 2001; Kim et al., 2007; Shin et al., 2017; Frank et al., 2018)) also alters WUE. Together, this
262 suggests that the entire circadian oscillator influences WUE, and that alterations in water
263 use that are caused by mutations to the circadian oscillator are not confined to a specific

264 sub-loop of the circadian oscillator or restricted to its input or output pathways. One
265 explanation for these circadian-system wide alterations in WUE relates to the nature of
266 feedback within the circadian oscillator. The complex feedback and interconnectivity of the
267 circadian oscillator means that individual components of the circadian oscillator that directly
268 influence stomatal function or water use are likely to be altered by mutations that are distal
269 to that component. Therefore, if correct circadian timing is required for optimum water use
270 efficiency, multiple components of the circadian oscillator are likely to influence water use
271 efficiency.

272 The sugar signalling proteins TPS1 and KIN10 influence a broad range of phenotypes, in
273 addition to participating in circadian entrainment (Baena-González et al., 2007; Gómez et al.,
274 2010; Paul et al., 2010; Delatte et al., 2011; Shin et al., 2017; Frank et al., 2018; Nietzsche
275 et al., 2018; Simon et al., 2018). The *tps1*-12 TILLING mutant of TPS1 decreases stomatal
276 aperture and increases the ABA sensitivity of guard cells (Gómez et al., 2010), whereas we
277 found that *tps1*-11 and *tps1*-12 had lower long-term WUE than the wild type (Fig. 1).
278 Reduced biomass accumulation in *tps1*-11 and *tps1*-12 (Fig. 2A) was consistent with slow
279 growth of these alleles (Gómez et al., 2010). Overall, this suggests that the decreased
280 stomatal aperture of *tps1*-12 mutants does not translate into an overall increase in WUE,
281 potentially due to slower growth of the *tps1* mutants (Fig. 2A) (Gómez et al., 2010). The
282 broad range of phenotypes that are altered in *tps1*-11, *tps1*-12 and KIN10-ox 6.5 indicates
283 that these genotypes might alter WUE through mechanisms other than circadian regulation.

284 *Potential roles for the evening complex in WUE*

285 Our finding that ELF3 is important for WUE (Fig. 1) is supported by previous evidence.
286 Under constant light conditions, wild type Arabidopsis has circadian rhythms of stomatal
287 aperture, whereas *elf3* stomata are constantly open and unresponsive to light and dark
288 (Kinoshita et al., 2011). Furthermore, ELF3 negatively regulates blue light-mediated stomatal
289 opening (Kinoshita and Hayashi, 2011). Therefore, perturbation of the anticipation of

290 day/night transitions or responses to environmental cues in *elf3* stomata might cause long-
291 term alterations in WUE.

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

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

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

312 *Multiple physiological causes of altered WUE in circadian oscillator mutants*
313 Our data suggest that changes in WUE caused by misexpression of circadian clock
314 components might be due to a combination of physiological factors. Some mutants or
315 overexpressors tested alter biomass accumulation, whilst others predominantly alter water

316 loss (Fig. 2), so mutations to the circadian oscillator did not alter water use by specifically
317 altering one of carbon assimilation or transpiration. This is consistent with previous work
318 demonstrating that both stomatal opening and CO_2 fixation is perturbed in circadian
319 arrhythmic plants under light/dark cycles (Dodd et al., 2005), and with the findings that daily
320 carbohydrate management is dependent upon correct circadian regulation (Graf et al.,
321 2010). We speculate that delayed or advanced stomatal and photosynthetic responses to
322 the day-night cycle might occur in circadian period mutants, because period mutants
323 inaccurately anticipate the onset of dawn (Dodd et al., 2014). Circadian clock mutants might
324 also affect WUE by changing the sensitivity of stomatal movements and photosynthesis to
325 environmental transitions, because there is circadian gating of the responses of both
326 stomata and photosynthesis to environmental cues (Dodd et al., 2006; Kinoshita et al., 2011;
327 Litthauer et al., 2015; Joo et al., 2017; Cano-Ramirez et al., 2018). Some effects of the
328 circadian oscillator upon WUE arise from alterations in leaf size that occur in some circadian
329 oscillator mutants (Fig. 3A, B). This suggests that developmental alterations arising from
330 lesions in the circadian oscillator can lead to changes in WUE. Such developmental
331 alterations might alter WUE by changing airflow around the rosette, boundary layer
332 conductance, or internal leaf structure.

333 *Conclusions*

334 We show that circadian regulation contributes to whole plant long-term WUE under cycles of
335 day and night. This control occurs partly through the influence of the circadian oscillator
336 upon rosette architecture. Mutation or overexpression of CCA1, TOC1, ELF3, GI, GRP7,
337 PRR5, PRR7, PRR9, TEJ and ZTL altered WUE under our experimental conditions. The
338 roles of these genes in WUE may be independent or overlapping, and their WUE
339 phenotypes might be due to direct effects of these genes, or indirect effects on transcript
340 and/or protein abundance of other circadian clock gene(s). Misregulation of the expression
341 of CHE, FKF1, LKP2, RVE4, RVE8, PRR3, ELF4, LUX and WNK1 did not appear to alter
342 WUE under our experimental conditions.

343 Our results have a number of broad implications. Firstly, our data suggest that alterations in
344 circadian function that arise during crop breeding could have the potential to increase or
345 decrease WUE. Therefore, manipulation of the functioning of the circadian oscillator might
346 represent a pathway to tune the WUE of crops. Second, our results indicate that circadian
347 regulation in a single cell type can have implications for whole-plant physiology. Finally, our
348 findings suggest that circadian regulation can alter a single trait (WUE) by affecting many
349 aspects of physiology. In future, it would be informative to distinguish the contribution to
350 overall WUE of circadian regulation within additional cell types, such as the mesophyll,
351 vascular tissue, and root cell types.

352

353 **Materials and methods**

354 *Plant material and growth conditions*

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

370 *Generation of transgenic lines*

371 To create the *GC1::CCA1:nos* (*GC*), *GC1::TOC1:nos* (*GT*), *MYB60::CCA1:nos* (*MC*) and
372 *MYB60::TOC1:nos* (*MT*) constructs, the CaMV *nos* terminator sequence was ligated
373 between the SpeI and NotI restriction sites in the pGREENII0229 binary vector (Hellens et
374 al., 2000). The *GC1* upstream sequence (-1894 to -190) or *MYB60* upstream sequence (-
375 1724 to -429) was then ligated between the KpnI and ApaI restriction sites of
376 pGREENII0229. Finally, the *CCA1* coding sequence or *TOC1* coding sequence, obtained
377 using RT-PCR, was ligated between the restriction XhoI and XmaI sites. Primers used are
378 identified in Table S2. Constructs were transformed into Col-0 wild type Arabidopsis using
379 transformation with *Agrobacterium tumefaciens* strain GV3101. Transformants were
380 identified by screening for phosphinothricin resistance, then further validated using genomic
381 DNA PCR. Homozygous lines were identified via phosphinothricin (BASTA) resistance, and
382 two independently transformed homozygous lines were investigated in detail per genotype.

383 Guard cell specificity of promoter activity was investigated using *GC1::GFP:nos* and
384 *MYB60::GFP:nos* promoter-reporter lines (Sup. Fig. 3A-C), which were created as above
385 with the *GFP* coding sequence ligated between the XhoI and XmaI restriction sites. Leaf
386 discs (5 mm diameter) from seedlings or mature plants were mounted on microscope slides
387 with dH_2O, and examined for GFP fluorescence using confocal microscopy (Leica DMI6000).
388 The following settings were used: argon laser at 20% capacity, 488 nm laser at 48%
389 capacity with a bandwidth of 505 nm–515 nm, gain of 1250, offset at 0.2%, 20x or 40x
390 objective, zoom x1 to x4.

391 *Measurement of water use efficiency*

392 The WUE assay was adapted from Wituszynska et al. (2013) (Wituszyńska et al., 2013).
393 Plants were grown for 6 weeks in modified 50 ml Falcon tubes. The Falcon tube systems
394 consisted of a 50 ml Falcon tube filled with 37.5 ml of a 1:1 ratio of compost: perlite and 35
395 ml of Milli-Q water (Merck), with the remaining volume filled with a 1:1 ratio of compost: Milli-
396 Q water (Fig. S3). Each Falcon tube lid had a 2 mm diameter hole drilled in its centre to

397 allow plant growth. The lid was spray-painted black (Hycote) because we found that the
398 orange colour of the Falcon tube lid caused leaf curling (Fig. S3). The system was wrapped
399 in aluminium foil to exclude light (Fig. S3). 10-15 seeds were sown through the Falcon tube
400 lid using a pipette. Following stratification, Falcon tube systems were placed under growth
401 conditions using a randomised experimental design. 7 days after germination, seedlings
402 were trimmed to one per Falcon tube system, and initial Falcon tube weight was recorded.
403 After 6 weeks of growth, rosette leaf surface area was measured by photography (D50;
404 Nikon) and Fiji software, rosette dry weight was measured (4 d at 60°C), and final Falcon
405 tube weight was recorded. Negative controls (Falcon tube systems without plants) were
406 used to assess soil water evaporation over 18 experimental repeats, with an overall mean
407 weight loss of 0.513 g ± 0.004 g over 6 weeks for plant-free Falcon tubes.

408 Plant WUE was calculated as follows:

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

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

418 *Dehydration tolerance assay*

419 This assay was adapted from Legnaioli et al. (2009). For experiments investigating survival
420 to dehydration, surface-sterilized seeds were sown on Petri dishes containing half strength
421 Murashige & Skoog basal salt mixture (0.5x MS) (Duchefa), supplemented with 0.8% (w/v)

422 agar and 3% (w/v) sucrose, then stratified for 3 days at 4 °C before transfer to the growth
423 chamber. For these experiments, seedlings were cultivated in MLR-352 growth chambers
424 (Panasonic) at 19°C with photon flux density of 120 µmol m^{-2} s^{-1}. 14-day old seedlings were
425 dehydrated on a double layer of filter paper (Fisher Scientific) for 9 h under constant light
426 conditions, watered with sterile dH_2O, and kept under constant light conditions for a further
427 48 h before being scored for survival. Seedlings with a green apical meristematic region
428 were counted as survivors. 32 seedlings were treated per genotype and within each
429 experimental repeat.

430 *Measurement of stomatal density*

431 Plants were grown for 7-8 weeks on compost mix. Dental paste (Coltene) was applied to the
432 abaxial surface of fully expanded leaves. Transparent nail varnish (Rimmel) was applied to
433 these leaf moulds once they had set, and then peeled away from the mould using clear
434 adhesive tape (Scotch Crystal). Stomatal and pavement cells were counted within an
435 800 µm x 800 µm square at the centre of each leaf half, using an epifluorescence
436 microscope (HAL100; Zeiss) and Volocity (Perkin Elmer) and Fiji software. For each
437 experimental repeat, two leaves were sampled per plant and eight plants sampled per
438 genotype. Stomatal index was calculated as follows:

$$SI = \frac{s}{s+p} * 100$$

439 Where *SI* is the stomatal index, *s* the number of stomata in the field of view (800 µm x
440 800 µm), and *p* the number of pavement cells in the field of view.

441 *RNA extraction and qRT-PCR*

442 RNA extractions, cDNA synthesis, and qRT-PCR were performed according to (Simon et al.,
443 2018), except approximately 10 seedlings were used per RNA sample and analysis was
444 performed using an MXPro 3005 real time PCR system (Agilent) with 5x HOT FIREPol
445 EvaGreen qPCR mastermix (Solis Biodyne). qRT-PCR primers are provided in Table S3.
446 Rhythmic features within qPCR data were identified using the BioDare2 platform (Zielinski et

447 al., 2014), using the Fast Fourier Transform Non-Linear Least Squares method (FFT-NLLS).
448 One independently-transformed line of each guard cell-specific circadian clock gene
449 overexpressor was also investigated using qRT-PCR conducted on RNA isolated from
450 epidermal peels. Abaxial leaf epidermis was detached, then washed in 10 mM MES (pH
451 6.15, adjusted using 10 M KOH) to remove RNA derived from ruptured epidermal cells. Each
452 RNA sample was derived from 20 epidermal peels (five plants, four leaves per plant) that
453 were collated and flash-frozen in liquid nitrogen. Guard cell RNA was extracted using the
454 RNeasy UCP Micro Kit (Qiagen) according to manufacturer's instructions, with the following
455 modification: guard cell lysis was performed by adding glass beads (425 µm - 600 µm
456 diameter, acid washed, from Sigma-Aldrich) and 350 µl RULT buffer to the sample, then
457 vortexed for 5 min.

458 *Accession numbers*

459 Arabidopsis Genome Initiative identifiers for the genes mentioned in this study are: *CCA1*
460 (*CIRCADIAN CLOCK ASSOCIATED1*, At2g46830), *CHE* (*CCA1 HIKING EXPEDITION*,
461 At5g08330), *ELF3* (*EARLY FLOWERING3*, At2g25930), *ELF4* (*EARLY FLOWERING4*,
462 At2g40080), *FKF1* (*F BOX1*, At1g68050), *GI* (*GIGANTEA*, At1g22770), *GRP7* (*GLYCINE*
463 *RICH PROTEIN7*, At2g21660), *KIN10* (*SNF1-RELATED PROTEIN KINASE1.1*, At3g01090),
464 *LKP2* (*LOV KELCH PROTEIN2*, At2g18915), *LUX* (*LUX ARRHYTHMO*, At3g46640),
465 *MYB60* (*MYB DOMAIN PROTEIN60*, At1g08810), *PRR3* (*PSEUDO-RESPONSE*
466 *REGULATOR3*, At5g60100), *PRR5* (*PSEUDO-RESPONSE REGULATOR5*, At5g24470),
467 *PRR7* (PSEUDO-RESPONSE REGULATOR7, At5g02810), *PRR9* (*PSEUDO-RESPONSE*
468 *REGULATOR9*, At2g46790), *RVE4* (*REVEILLE4*, At5g02840), *TEJ* (*POLY(ADP-*
469 *RIBOSE)GLYCOHYDROLASE1*, At2g31870), *TIC* (*TIME FOR COFFEE*, At3gt22380),
470 *TOC1* (*TIMING OF CAB EXPRESSION1*, At5g61380), *TPS1* (*TREHALOSE-6-PHOSPHATE*
471 *SYNTHASE1*, At1g78580), *WNK1* (*WITH NO LYSINE KINASE1*, At3g04910), *ZTL*
472 (*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.

484 **Figure legends**

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

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

510 **Figure 3.** The circadian oscillator alters WUE partially by changing rosette architecture. (A)
511 Altering circadian-associated gene expression can affect rosette architecture and size, as
512 illustrated for *elf3*-1, *lux*-1, and *gi*-2 in (Col-0 background). Image backgrounds removed for
513 clarity. Variation in rosette leaf surface area across the genotypes investigated explained (B)
514 16% of variation in WUE ($p < 0.001$, $r = 0.400$, $r^2 = 0.160$), (C) 83% of variation in
515 transpiration ($p < 0.001$, $r = 0.912$, $r^2 = 0.832$) and (D) 73% of variation in rosette dry
516 biomass ($p < 0.001$, $r = 0.857$, $r^2 = 0.734$). Data were analysed using Pearson correlation
517 tests.

518 **Figure 4.** Overexpressing *CCA1* or *TOC1* in guard cells affects WUE and survival of
519 dehydration by seedlings. (A) Constructs used to overexpress *CCA1* or *TOC1* coding
520 sequence under control of *GC1* or *MYB60* promoters. (B) Guard cell *CCA1* overexpression
521 can increase WUE. WUE expressed as a percentage of the wild type (normalised to 100%,
522 red reference line). Two to four independent experimental repeats were performed, with data
523 from one representative dataset shown (n = 5 - 15). Data for CCA1-ox and TOC1-ox are
524 derived from Fig. 1, for purposes of comparison. Data were analysed with independent
525 samples t-tests, and statistical significance compared to Col-0 is indicated using starring (**
526 = $p < 0.01$; *** = $p < 0.001$). (C) Guard cell *CCA1* or *TOC1* overexpression alters
527 dehydration survival of seedlings compared with the wild type. Data were obtained from
528 three independent experimental repeats (mean; n = 32 per experimental replicate; at least
529 two independent experimental repeats were performed for each genotype). A single
530 *GC1::TOC1* line is shown here because other lines produced extremely variable data. (D,E)
531 Guard cell *CCA1* or *TOC1* overexpression does not affect (D) stomatal index nor (E)
532 stomatal density. Two independent experimental repeats were performed, with data from
533 one representative dataset shown (n = 19 - 32; mean ± S.E.M.). Data were analysed with
534 ANOVA and Tukey's post hoc tests (NS = $p > 0.05$). Bar colours identify the whole plant
535 overexpressor control (black), wild type control (dark grey), and guard cell-specific
536 overexpressor genotypes (light grey).

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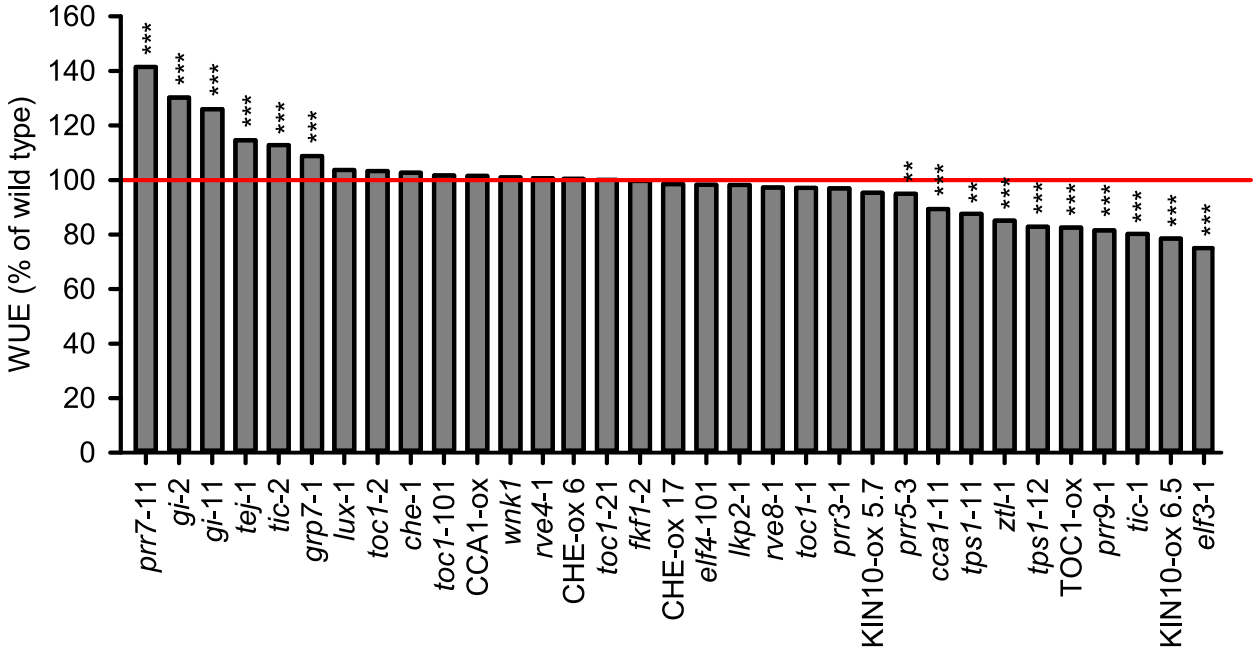

Figure 1. The circadian clock regulates long-term water use efficiency of Arabidopsis under light/dark cycles. The WUE of circadian clock mutants and overexpressors is expressed as a percentage of their respective background (normalized to 100%, red reference line) to account for WUE variation between 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.

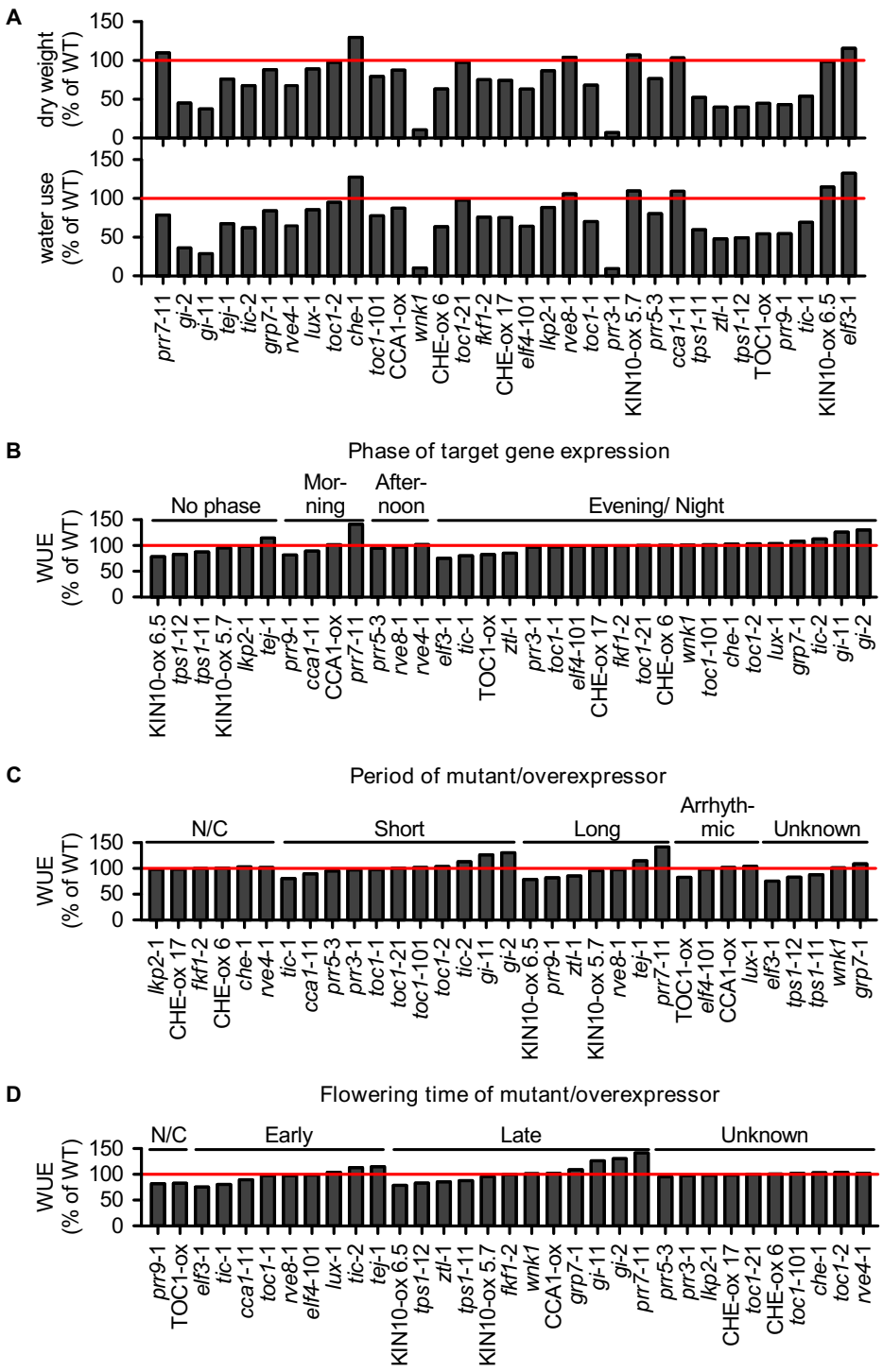

Figure 2. Altered WUE of plants with mutations or overexpression of circadian clock associated genes is not caused consistently by variation in one of dry weight, water use, phase of expression of each gene, or resultant altered period or flowering time. Data are derived from Fig. 1 and expressed as 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.

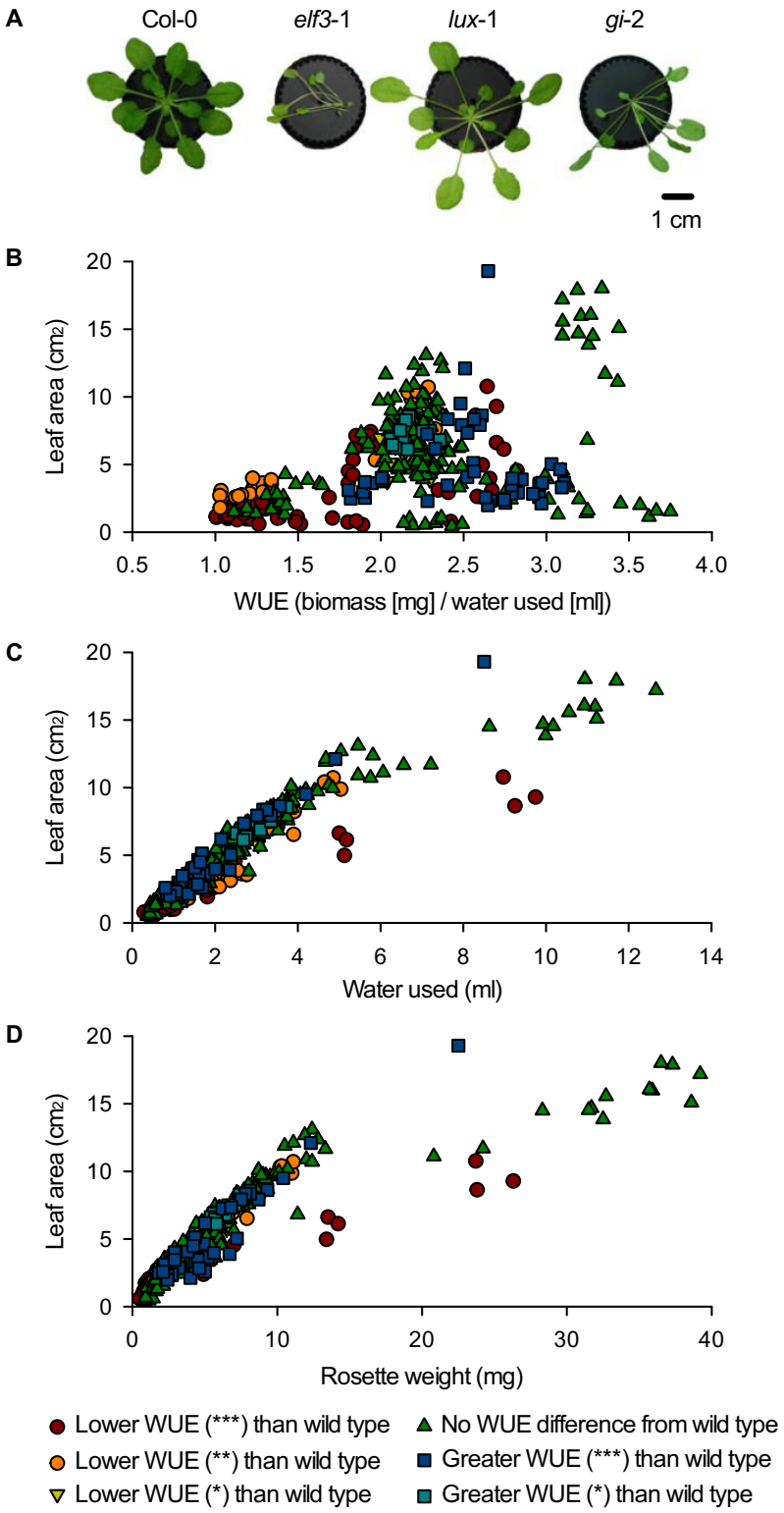

Figure 3. The circadian oscillator alters WUE partially by changing rosette architecture. (A) Altering circadian-associated gene expression can affect rosette architecture and size, as illustrated for *elf3*-1, *lux*-1, and *gi*-2 in (Col-0 background). Image backgrounds removed for clarity. Variation 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.

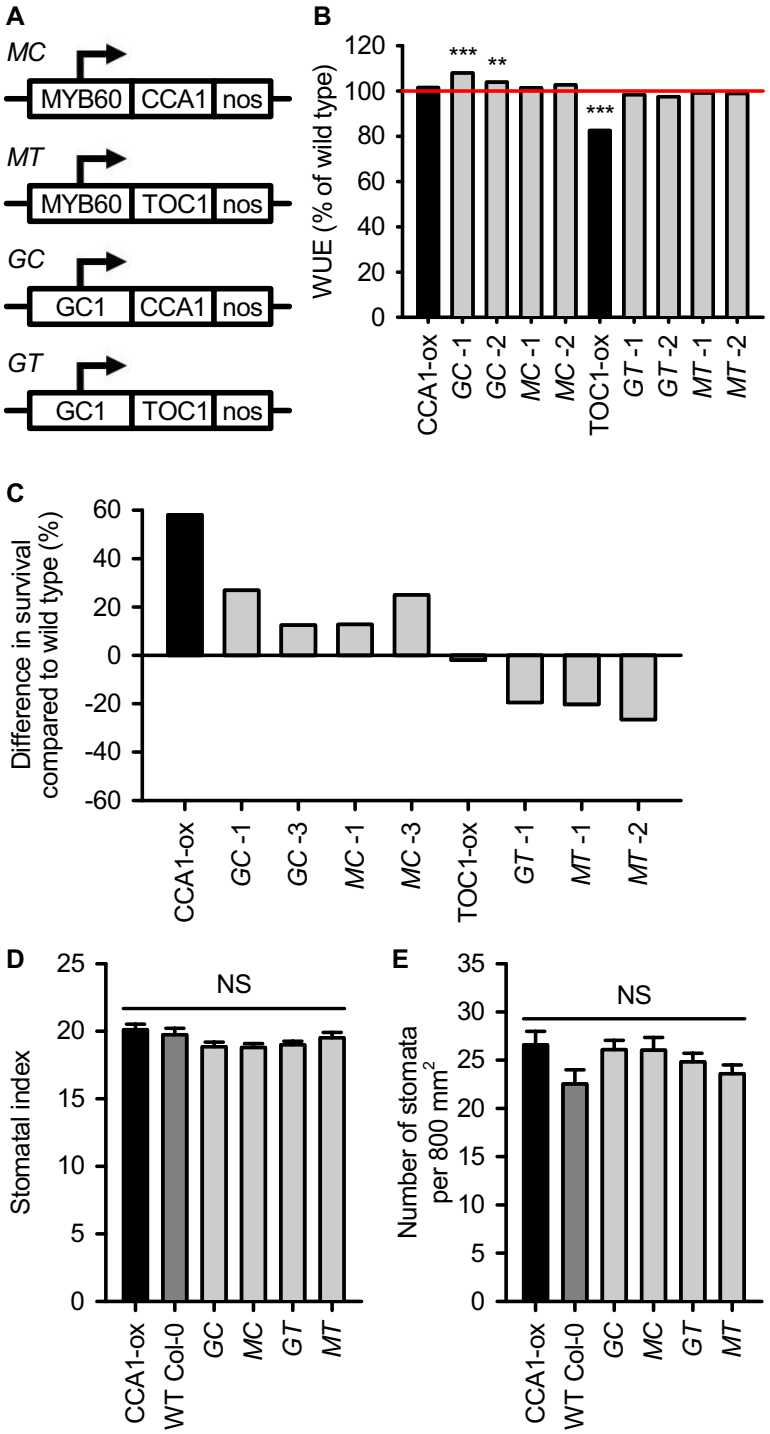

Figure 4. Overexpressing *CCA1* or *TOC1* in guard cells affects WUE and survival of dehydration by seedlings. (A) Constructs used to overexpress *CCA1* or *TOC1* coding sequence under control of *GC1* or *MYB60* promoters. (B) Guard cell *CCA1* overexpression can increase WUE. WUE expressed as a percentage 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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