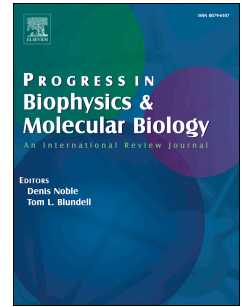


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Circadian leaf movements facilitate overtopping of neighbors

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1 **Circadian Leaf Movements Facilitate Overtopping of Neighbors**

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Abstract

Many plants exhibit circadian clock-driven leaf movements whereby the leaves are raised during the day to achieve a relatively high angle during the evening, before lowering late in the night. Such leaf movements were first recorded over 2,000 years ago but there is still much debate as to their purpose. We investigated whether such leaf movements within *Arabidopsis*, a ruderal rosette plant, can aid in overtopping leaves of neighboring plants. Wild type and circadian clock mutant plants were grown in an alternating grid system so that their leaves would meet as the plants grew. Experiments were performed using day lengths that matched the endogenous rhythm of either wild type or mutant. Plants grown in a day length shorter than their endogenous rhythm were consistently overtopped by plants which were in synchrony with the day night cycle, demonstrating a clear overtopping advantage resulting from circadian leaf movement rhythms. Furthermore, we found that this leaf overtopping as a result of correctly synchronized circadian leaf movements is additive to leaf overtopping due to shade avoidance. Curiously, this did not apply to plants grown in a day length longer than their endogenous period. Plants grown in a day length longer than their endogenous period were able to adapt their leaf rhythms and suffered no overtopping disadvantage. Crucially, our results show that, in a context-dependent manner, circadian clock-driven leaf movements in resonance with the external light / dark cycle can facilitate overtopping of the leaves of neighboring plants.

40

Key Words

Circadian, hyponasty, leaf movement, overtopping

43

44 **1. Introduction**

45 Many plants exhibit circadian clock-driven leaf movements whereby the leaves are raised during the
46 day to achieve a high angle during the evening before lowering late in the night. Diurnal leaf
47 movements were first observed from c.324 BCE by Androstheneas (scribe to Alexander the Great) in
48 the tamarind tree, *Tamarindus indica* (Hort, 1916). In 1729, the astronomer De Mairan conducted a
49 series of experiments in which he placed plants into continuous darkness. When he observed the
50 leaves opening in the morning he correctly deduced that an endogenous biological timing mechanism
51 must be in operation (De Mairan, 1729). Under constant conditions, the period of these oscillations is
52 approximately 24 hours and forms a classical example of circadian rhythmicity, a phenomenon which
53 regulates a wide range of processes in plants, animals and microbes including our own sleep/wake
54 cycles (Young and Kay, 2001). Leaf movement assays have recently formed a key marker for the
55 observation of plant circadian rhythms and have been central to the discovery of a number of the
56 molecular components of the plant central clock mechanism (Hicks et al., 1996; Schaffer et al., 1998;
57 Wang & Tobin, 1998), yet their ecological function remains the subject of much debate.

58 Circadian leaf movements do not always involve the raising of leaves. Darwin observed a lowering
59 leaves to a vertical orientation at night in beans (Darwin, 1880). He was the first to suggest a
60 functional role for what he called the sleep movements of leaves, with his hypothesis that leaves held
61 vertically would radiate less heat at night than horizontally held leaves and so such movements might
62 protect plants from the effects of chilling or frost damage through the conservation of heat (Darwin,
63 1880). Experiments, which Darwin conducted on a number of plant species including *Trifolium* and
64 *Oxalis*, revealed that leaves forced into a horizontal position during periods of freezing temperature at
65 night suffered more frost damage than leaves that were allowed to assume a normal vertical
66 orientation (Darwin, 1880). However, Schwintzer (1971) suggested that Darwin's findings were
67 probably artefactual after observing that vertical orientation of leaves at night failed to provide
68 adequate protection from freezing in soybeans as only small (<1°C) temperature differences were
69 revealed to exist between leaves held vertically and those held horizontally. Enright (1982) replicated
70 Schwintzer's finding of only a small temperature difference between horizontally and vertically held
71 leaves, but also found that, under non-freezing conditions, warmer vertically oriented leaves grew
72 slightly more rapidly than horizontally oriented ones, indicative of a possible role for circadian leaf
73 movements as a protective mechanism against chilling as opposed to freezing in certain plants.

74 However, all such experiments investigated plants showing large changes in leaf orientation from
75 horizontal during the day to near vertical orientation of leaves at night. These changes are the result
76 of the action of specific organs called pulvini, particularly common in legumes, which raise or lower
77 leaves as a result of changes in turgor pressure (Koller et al., 2000). In plants which lack pulvini leaf
78 movements are simply the result of alternating abaxial and adaxial growth within the petiole as the
79 leaf grows (Koller et al., 2000). Generally, this results in a much more subtle leaf movement. None-
80 the-less, this phenomenon has been demonstrated to result some frost protection in giant rosette
81 plants such as *Senecio keniodendron* which inhabit tropical mountains. These plants hold their upper
82 leaves at a very high angle during the day but raise them still further to form a closed “night-bud”
83 during the night (Beck et al., 1982; Smith, 1974). In contrast, rosette plants such as dandelion or
84 *Arabidopsis* which inhabit more temperate regions, raising their leaves from horizontal during the day
85 to a maximum inclination no more than 45 degrees during the night (Mayer 1966; Millenaar et al.,
86 2005). Such an occurrence would not seem likely to provide any degree of frost protection and the
87 reason for such leaf movements is unexplained. We propose here that the circadian leaf movements
88 in such rosette plants could have an additional benefit as a mechanism for facilitating overtopping of
89 neighboring plants. These plants show a ruderal strategy and are generally early colonizers of open
90 ground in spring, quickly producing seed (Pigliucci, 2002). Competition for light amongst such species
91 is intense and such overtopping would be an advantage in preventing shading. In order to investigate
92 this hypothesis, we chose the model plant, *Arabidopsis*. As mentioned, *Arabidopsis* features a
93 characteristic rhythmic leaf movement that is believed to be based upon differential growth of the
94 petioles (Engelmann and Johnsson, 1998). A circadian rhythm controls the elongation rates of abaxial
95 and adaxial cells in turn in order to drive the positioning of leaves (Polko et al., 2012; Rauf et al.,
96 2013). In *Arabidopsis*, as in other plants, leaves are raised during the day and fall again at the end of
97 the night, a pattern of oscillation which continues following transfer to constant light (Hicks et al.,
98 1996). High resolution, automated analysis of this phenomenon using near-infrared laser scanning has
99 additionally revealed that leaf/petiole elongation in *Arabidopsis* also shows a circadian regulation of
100 leaf elongation growth, with the peak of leaf/petiole elongation occurring in the morning just as
101 leaves are rising (Dornbusch et al., 2014), further enhancing the proposition that leaf movement
102 rhythms may be part of an overtopping mechanism. In our assay, we set up a series of experiments
103 where wild type plants were placed in close proximity with a range of circadian clock mutants in a

104 range of day lengths. We were able to show that circadian clock-driven leaf movements in resonance
105 with the external light / dark cycle do facilitate overtopping of neighboring plants which fail to show
106 correct timing of these movements; thus, supporting our hypothesis. Furthermore, we show that this
107 overtopping mechanism acts additively with overtopping due to shade avoidance.

108

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109 2. Materials and Methods

110 2.1. Plant Materials and Growth Conditions

111 The *CCA1* overexpressor (*CCA1ox*) line o34 and *ztl* mutant (*ado1-1*), both in the Columbia ecotype,
112 and *toc1-1* in the C24 ecotype were previously described (Wang & Tobin, 1998; Strayer et al., 2000;
113 Jarillo et al., 2001). Seeds were sprinkled onto a moist compost comprised of John Innes No 3 (ICL
114 Levington, UK), Levington M3 (ICL Levington M3) and Perlite (Sinclair, UK) mixed in a ratio of 6:6:1.
115 Seedlings were germinated in 16 hour light / 8 hour dark cycles. After 7 days seedlings were
116 transplanted individually as described below and maintained in 16 hour light / 8 hour dark cycles for
117 another 3 days before transfer to the experimental conditions.

118 All experiments were performed at 21 °C. 16 hour white light / 8 hour dark cycles were generated in a
119 growth room fitted with Osram 840 cool white fluorescent tubes (Osram, UK), providing of 150 μmol
120 $\text{m}^{-2} \text{s}^{-1}$ photosynthetically-active radiation (PAR) and a red: far red ratio (R:FR) of 5.1. Non-24 hour day
121 length cycles were generated in a Fytoscope FS 80-RGBIR Mini cabinet (Photon Systems International,
122 Brno, Czech Republic) using an equal mix of red (630 nm), green (530 nm) and blue (470 nm) LEDs,
123 providing 150 $\mu\text{mol} \text{m}^{-2} \text{s}^{-1}$ PAR and a R:FR of 5.1. White light supplemented by far red light (FR) was
124 generated in the cabinets as described in Wang et al (2011), providing 50 $\mu\text{mol} \text{m}^{-2} \text{s}^{-1}$ PAR and a R:FR
125 of 1.0. All light measurements were made using a StellarNet EPP2000-HR spectroradiometer.

126

127 2.2. Proximity Experiments

128 Wild-type seedlings were transplanted in an alternating grid pattern with mutant lines. A combined
129 total of 24 wild type and mutant seedlings were planted alternately in four 6 x 4 grids with 4 cm
130 between plants. Leaf overtopping between neighbors was recorded along both the ranks and files. For
131 this, the number of overlaps won by each genotype was counted. An overlap was defined as a leaf
132 covering any part of a leaf of the opposing genotype. Overlaps were counted just prior to flowering
133 when the first bud became visible on any plant. Overlaps were counted within the final two hours of
134 the light period. Rosette radius was measured by measuring the radius of the smallest possible circle
135 which could be positioned so as to encompass the tips of the three longest leaves on its perimeter.
136 Each experiment was repeated between two and four times.

137

138

139 *2.3. Analysis of leaf movement rhythms*

140 Time lapse photography was used to record leaf angles of two-week old seedlings. Digital images
141 were collected at 2 hour intervals for three days using TeckNet C016 USB HD 720P webcams and
142 boroo WebCam2 software (Lumai HB, Sweden) or iSpy software (iSpyConnect, Australia). Cameras
143 were placed at soil level. Leaf angle between the horizontal and a line from the leaf tip to the petiole
144 origin was measured using Image-J (NIH, USA). All angles were adjusted to account for azimuth by
145 applying a multiplier based on measurement of the angle subtended on the image by the known right
146 angle between the zenith and horizontal soil surface at the same azimuth as the leaf. Image capture
147 commenced at 7 days after transfer to experimental conditions. Leaf tip position for a sample of
148 between 6 and 10 leaves was recorded for 3 days for each genotype.

149

150 **3. Results**

151 In order to examine whether circadian leaf movements enhance overtopping of the leaves of
152 neighboring plants, we placed wild type plants in direct proximity with a range of circadian clock
153 mutants in a range of light / dark cycles corresponding to the endogenous period lengths of either the
154 wild type or mutant lines. Plants were grown in an alternating grid arrangement so that opposing
155 genotypes came into direct physical contact. The outcome was measured in terms of leaf overtopping
156 events whereby the leaf of one genotype overtopped any part of a leaf of an opposing genotype along
157 the ranks and files of the grid.

158

159 *3.1. Interaction between wild type and a long period mutant*

160 In our initial experiments, wild type plants were grown in direct proximity to the long period *ztl* long
161 period mutant, which displays a 27 hour period (Somers et al., 2000). In light-dark cycles of 16 hours
162 light / 8 hours dark, resonating with the wild type period length of 24 hours, leaves of wild type plants
163 overtopped those of *ztl* in over 71% of cases on average (Fig. 1A) (p value, chi squared test, 7.38×10^{-8}
164 for a null hypothesis of no significant difference). Examination of leaf angles over a three day period

165 revealed that the leaf movement rhythms of both wild type and *ztI* showed a repeating 24 hour
166 pattern. This is indicative of the action of light in entrainment of the normally long period *ztI* line to
167 the external light / dark cycle. However, *ztI* leaf movement rhythms showed a consistent difference in
168 phase relative to those of wild type seedlings suggesting an imperfect entrainment (Fig. 1B).
169 Calculation of the mean phase of peak hyponasty, expressed as the time after dawn, revealed that
170 wild type showed a mean peak phase at 12.22 h, while *ztI* showed a mean peak phase at 13.73 h (Fig.
171 2) (p value, heteroscedastic T-test, 0.018).

172 In light-dark cycles of 18 hours light / 9 hours dark, resonating with the *ztI* period length of 27 hours,
173 we observed that wild type and *ztI* fared equally well with respect to leaf overlaps despite the lack of
174 resonance between the external light / dark cycle and the endogenous period of wild type seedlings
175 (Fig. 3A). As in 24 hour days, leaf movement rhythms of both genotypes were able to entrain to the
176 light / dark cycle but, crucially, wild type seedlings were able to entrain almost perfectly to the longer,
177 driven period, showing no significant difference in peak times from those of *ztI* (Fig. 2, 3B). Mean
178 phase of peak hyponasty in this non 24-hour day was corrected to represent the proportion of a
179 standard 24 hour day / night cycle at which the peaks occurred. This allows direct comparison with
180 peak times of these genotypes in the 24 hour days and 27 hour days which revealed that both
181 genotypes showed a relatively earlier peak leaf angle with respect to the total duration of the day
182 when grown in 27 hour days.

183

184 3.2. Interaction between wild type and a short period mutant

185 Wild type seedlings were then grown in direct proximity to a short period mutant, *toc1*, which
186 displays an endogenous period of 21 hours (Strayer et al., 2000). When wild type seedlings were
187 placed alongside *toc1* in 21 hour days of 14 hours light / 7 hour dark cycles, we observed that *toc1*
188 showed a greater percentage of overlaps won than wild type (62% vs 38%; p value, chi squared test,
189 0.017 for a null hypothesis of no significant difference) (Fig. 4A). Once again, seedlings of the
190 genotype adapting to a day length shorter than its endogenous period, showed an inability to
191 properly entrain their leaf movements to these conditions. In this case, the wild-type seedlings
192 adapted well to a 21 hour day whereas previously this same behavior was exhibited when the long
193 period *ztI* mutant which had adapted well to a 24 hour day. Both wild type and *toc1* seedlings showed

194 an apparent 21 hour repeating pattern length of leaf movement but wild type seedlings showed a
195 delayed phase (Fig. 4B). For phase analysis in these 21 hour days, mean phase of leaf position was,
196 again, corrected to represent the proportion of a standard 24 hour day / night cycle at which the
197 peaks occurred. In this case mean trough phase was plotted as peak phase for some seedlings
198 occurred at or beyond dusk. With leaf position beyond dusk being obscured due to absence of light
199 beyond dusk, it was not possible to be certain of the exact peak phase for such seedlings. In this case,
200 trough phase was plotted rather than peak phase as peak time was not always clear for every
201 seedlings due to the peak being on the border of dusk, particularly for wild type seedlings. Mean
202 trough phase for *toc1* seedlings occurred at 6.53 h after dawn, while mean trough phase for wild type
203 seedlings occurred at 8.09 h (Fig. 5) (p value, heteroscedastic T-test, 0.011). As previously, mean
204 phase of peak hyponasty in this non 24-hour day was corrected to represent the proportion of a
205 standard 24 hour day / night cycle at which the peaks occurred.

206 When wild type and *toc1* seedlings were grown alongside each other in 24 hour day lengths of 16
207 hours light / 8 hour dark cycles, both genotypes fared equally well in terms of overtopping (Fig. 6A).
208 Similarly, there was no significant difference in phase of leaf movement rhythms between the two
209 genotypes (Fig. 5, 6B), indicating that *toc1* had entrained well to the longer 24 hour day length. As
210 with the *ztl* experiments, experiments with wild type and *toc1* seedlings suggest that seedlings of
211 *Arabidopsis* are better able to adapt and entrain to a day length longer than their endogenous period
212 than they are to adapt to a day length that is shorter. In addition, direct comparison with peak times
213 of these genotypes in the 24 hour days and 21 hour days revealed that both genotypes, once again,
214 showed a relatively earlier peak leaf angle with respect to the total duration of the day in the longer
215 of the two day lengths.

216

217 3.3. Interaction between wild type and an arrhythmic mutant

218 We then placed wild type plants in direct proximity to a well-characterized line overexpressing the
219 *CIRCADIAN CLOCK ASSOCIATED1* gene (*CCA1ox*) that is part of the central clock loop (Hsu and
220 Harmer). The *CCA1ox* line lacks any circadian clock-driven leaf movements (Wang et al., 1998). To our
221 surprise, in 24 hour days of 16 hours light / 8 hours dark, the *CCA1ox* line showed significantly more
222 overtopping of the opposing genotype than the wild type did (66% of overlaps won by *CCA1ox* vs 34%

223 won by wild type) (p value, chi squared test, 0.003 for a null hypothesis of no significant difference)
224 (Fig. 7A). Analysis of leaf tip traces in these conditions showed that *CCA1ox* showed a constitutive
225 extreme hyponasty (Fig. 7B), a feature shared with the only other unconditionally arrhythmic
226 circadian clock mutant, the *LATE ELONGATED HYPOCOTYL* overexpressor, *lhy-1* (Schaffer, 1997). Such
227 extreme hyponasty may account for the observed overtopping of wild type seedlings. *CCA1-ox* did,
228 however, show a minor environmentally-driven, non-anticipatory oscillation in leaf movement in
229 these light / dark cycles with leaf angle showing a slight fall during the light period and a slight rise
230 during the dark period (Fig. 7B) (leaf position begins each day slightly higher than it ended the
231 previous day).

232

233 3.4. Interaction in white light supplemented by far red

234 Thus far, our interaction experiments were carried out in white light lacking any significant amounts
235 of far red light (R:FR 5.1). Such conditions would greatly reduce the potential for the shade avoidance
236 response that would normally form an additional part of interactions between neighboring plants in
237 the wild. In natural light, depletion of red but not far red wavelengths in light reflected from
238 neighboring plants results in a decreased R:FR and causes increased hyponasty as well as leaf and
239 petiole elongation (Roig-Villanova and Martínez-García 2016) which might alter the importance of leaf
240 movement as an overtopping strategy. In order to examine whether leaf movement rhythms also
241 confer an overtopping advantage under more natural environmental conditions, we repeated the 24
242 hour day interaction experiments involving the *ztl* and *toc1* mutants in white light supplemented with
243 far red light, giving an R:FR of 1.0, similar to that of sunlight. Under these conditions, interaction
244 between wild type and long period *ztl* mutant seedlings, again, resulted in a significant advantage to
245 wild type seedlings in terms of overtopping events. In light-dark cycles of 16 hours light / 8 hours dark,
246 leaves of wild type plants overtopped those of *ztl* in over 69% of cases on average (Fig. 8A) (p value,
247 chi squared test, 1.55×10^{-7} for a null hypothesis of no significant difference). Analysis of leaf angle in
248 these seedlings revealed that, at all time points tested, leaf angle was considerably greater under
249 these conditions than in white light for both genotypes indicative of a marked shade avoidance
250 response (Fig. 8B). Nonetheless, diurnal leaf movement rhythms were still clearly visible in both wild
251 type and *ztl* mutant seedlings, demonstrating that the shade avoidance response did not obviate the
252 leaf movement rhythm. As in white light, leaf movement rhythms of both wild type and *ztl* showed a

253 24 hour pattern but with *ztl* leaf movement rhythms showing a consistent difference in phase relative
254 to those of wild type seedlings (Fig. 8B). Calculation of the mean phase of peak hyponasty revealed
255 that wild type showed a mean peak phase at 12.08 after dawn, while *ztl* showed a mean peak phase
256 at 14.58 h after dawn (Fig. 9A) (p value, heteroscedastic T-test, 1.20×10^{-7}). Thus, in light with a R:FR
257 equivalent to sunlight, *ztl* seedlings also showed an inability to properly entrain their leaf movements
258 to a day length shorter than their endogenous period and showed a reduced percentage of leaf
259 overlaps compared to wild type.

260 Interaction between wild type and short period *toc1* seedlings in 16 hours light / 8 hours dark cycles
261 using light with an R:FR of 1.0 resulted in no significant difference in the percentage of successful leaf
262 overlaps between the two genotypes (Fig. 10A). Thus, as in white light, no disadvantage was
263 conferred to a genotype with the shorter period length than the environmental light / dark cycles.
264 Again, however, clear leaf movement rhythms continued in both genotypes and, as in white light,
265 both genotypes showed no significant difference in phase and period of leaf movement rhythms (Fig.
266 9B, 10B) indicating that *toc1* had entrained well to the longer 24 hour day length. As with the *ztl*
267 experiment, however, leaf angle was greater at all time points in white light supplemented with far
268 red than in white light alone.

269 Thus, our experiments carried out in conditions allowing shade avoidance revealed that leaf
270 movement rhythms continue as in white light, albeit with a greater leaf angle. Our results in these
271 experiments also corroborate the findings in white light that seedlings of Arabidopsis are better able
272 to adapt and entrain to a day length longer than their endogenous period than they are to adapt to a
273 day length that is shorter. Crucially, as in white light, the inability to appropriately entrain leaf
274 movement rhythms to the environmental light / dark cycles was associated with a disadvantage in
275 terms of leaf overtopping.

276

277 4. Discussion

278 Darwin initiated research into the functional role of circadian clock-driven leaf movements over a
279 century ago (Darwin, 1880) and this role has subsequently been the subject of much speculation.
280 Here we illustrate a potential role for these movements in Arabidopsis as a mechanism of competition

281 for light. In the conditions of our study, we observed that circadian resonance of these leaf
282 movements can contribute to overtopping of the leaves of neighboring plants.

283 When plants were grown in a day length matching their own circadian period, their leaves
284 consistently overtopped those of a longer period mutant line. Wild type plants overtopped the long
285 period *ztl* mutant, in a 24-hour day. Similarly, the short period *toc1* mutant overtopped wild type in a
286 21-hour day, indicating that when environmental conditions were altered to suit these mutant
287 genotypes an overtopping advantage was conferred upon them. Crucially, plants of these “longer
288 period lines” showed a delayed phase of leaf movements in these conditions. We propose that the
289 correct timing of leaf movement ensures that leaves begin rising shortly after the commencement
290 elongation growth which is known to be triggered by light at dawn (Dornbusch et al., 2014). A delay in
291 raising leaves would mean that these leaves may be overtopped by the leaves of other plants.

292 The failure of “longer period lines” to sufficiently advance their rhythmic leaf movement as part of
293 entrainment to a shorter day length is consistent with the observations of Dornbusch et al. (2014)
294 who noted that an early dawn caused no advance in the leaf movement rhythm in wild type seedlings.
295 Significantly, though, the commencement of elongation growth did advance under these early dawn
296 conditions so as to begin at the new time of dawn (Dornbusch et al., 2014). Thus, the overtopping
297 effect that we observed would likely have a two-fold cause: in the “longer period lines”, elongation
298 growth would begin much earlier than leaf elevation and these leaves would be growing under
299 “control line” leaves at the same time as “control line” leaves would be growing over “longer period
300 line” leaves.

301 Conversely, no overtopping advantage was observed when plants grown in their own day length were
302 competed against shorter period lines. The *ztl* mutant fared equally with wild type when grown in 27-
303 hour days, while wild type fared equally with the *toc1-1* mutant line when grown in 24-hour days.
304 Here, we found that these “shorter period lines” were able to adapt to a day length longer than their
305 own endogenous period in terms of leaf movement. This is, again, consistent with the observations of
306 Dornbusch et al. (2014) who noted that a late dawn did cause a delay in leaf movement rhythm so
307 that both leaf movement and elongation growth remained well matched.

308 Another interesting observation was the occurrence of an earlier relative time of peak hyponasty in
309 longer day lengths. In 24 hour days versus 27 hour days (Fig. 2) or 21 hour days versus 24 hour days

310 (Fig. 5), all genotypes showed a relatively earlier peak leaf angle with respect to the total duration of
311 the day when grown in the longer day length. This difference is more than can be accounted for by a
312 simple determination of phase by dawn. Instead it appears that the preceding dusk may also play a
313 significant role in determining the phase exhibited in the following day.

314 Our study deals only with a mechanism for overtopping. A correctly-synchronized circadian clock
315 confers numerous such distinct advantages including correct starch usage patterns in plants (Graf et
316 al., 2010) and correct time of flowering (Song et al., 2015). However, several, more comprehensive
317 studies have already shown global fitness advantages. Yerushalmi et al. (2011) demonstrated that
318 evolutionary selection always favored lines whose circadian period resonated with the external
319 environment. Michael et al (2003) observed a latitudinal cline in the period of the *Arabidopsis*
320 circadian clock, suggesting that the clock plays a key role in adaptation to the local day / night cycle
321 (Michel et al., 2003). Dodd et al. (2005) also observed fitness advantages in monoculture experiments
322 where endogenous period resonated with the external light / dark cycle. However, consistent with
323 our observations, Dodd et al. (2005) noted that, in competition experiments between long period and
324 short period mutants, while a period length longer than that of the ambient light/dark cycle was
325 always disadvantageous in terms of the key traits of rosette diameter, fresh weight and dry weight, a
326 period length shorter than that of the ambient light/dark cycle was not always disadvantageous in
327 these respects. It is possible that overtopping of competitors' leaves may at least partly be
328 responsible for these experimental outcomes.

329 Surprisingly, the arrhythmic *CCA1ox* line gained an overtopping advantage over wild type plants in 24
330 hour light / dark cycles. It might be expected that the absence of leaf movements would be a
331 significant disadvantage in terms of overtopping. However, our assays demonstrated a consistently
332 high leaf angle in the arrhythmic line. Such a constitutive hyponasty would, no doubt, give a
333 considerable advantage in overtopping neighbors. The fact that such extreme hyponasty has not
334 evolved in wild type lines as an even better competitive strategy indicates that there must also be
335 considerable negative trade-offs associated with it. It is likely that a constitutively high elevation angle
336 will reduce light capture due to a reduced angle of incidence to the sun. These negative trade-offs
337 may, indeed, be part of the reason why *CCA1ox* plants show reduced net carbon fixation and,
338 ultimately, reduced aerial biomass when grown in monoculture in these same conditions (Dodd et al.,
339 2005). However, this issue does raise the issue of the wider implications of leaf movements. There

340 may be other positive or negative effects on light capture for example which we do not examine here.
341 We merely examine one aspect here, overtopping advantage, which will likely be part of a much
342 larger balanced equation of positive and negative impacts in the real environment in the ultimate
343 determinant of fitness.

344 Increased net carbon fixation and aerial biomass resulting from a light / dark cycle which resonates
345 with endogenous period (Dodd et al., 2005) will consistently have applied in our interaction studies.
346 However, overtopping only favored plants grown in a day length matching their own circadian period,
347 when they competed against a longer period line, not when they competed against a shorter period
348 line. This suggests that increased biomass is not a major contributor to the overtopping metric in our
349 observations as it did not simply always give overtopping advantage to the line with endogenous
350 period matching the external day length as occurs for biomass advantage (Dodd et al 2005). In our
351 experiments involving *ztl* or *toc1*, where we were competing lines of different period length against
352 each other, no significant differences in leaf length were observed between each pair of competing
353 genotypes (Table S1), nor were there any clear differences maximum and minimum leaf angles
354 different between these competing lines. Thus, our assay using an ordered grid, where competing
355 leaves meet tip to tip, leaf size / morphology advantages were not a factor. In theory, though, a
356 correctly timed leaf elevation could overtop another leaf irrespective of size differences. Indeed, it
357 could be equally beneficial in interspecific competition and in intraspecific competition. A larger leaf
358 moving through the same arc will generally be expected to have an advantage if the larger leaf were
359 to meet a smaller leaf tip-to-tip. In this case, the height moved at the tip of the larger leaf would be
360 greater. However, for a smaller leaf an advantage could still be gained through correctly timed leaf
361 movement if its tip were to meet the larger leaf at a point further in towards the stem of the larger
362 leaf. Such a phenomenon would be fairly common in a natural environment where competing plants
363 are arranged randomly rather than in an ordered grid.

364 Another aspect of the controlled environment nature of our study is the fact that our experimental
365 grid had an edge. Edge effects have been observed in a number of ecological situations and can result
366 in competitive advantages not being equally realized by all populations at the edge of a habitat. We
367 did not expect wild type and period length mutants to be differentially affected at the outer edge of
368 the grid versus the middle of the grid and, indeed, a calculation of overlaps for plants at the outer
369 edge versus the middle showed no difference in percentage competition won by each genotype

370 among plants at the edge of the grid. For example, wild type versus *toc1* in 24 hour days wild type
371 leaves won 50.5% of interactions, *toc1* 49.5% at the edges of the grid, almost identical to the pattern
372 seen for the grids as a whole in this assay.

373 Our study also shows that this overtopping mechanism is additive with respect to the increased
374 hyponasty-based overtopping mechanism observed as a result of shade avoidance. Leaf movement
375 rhythms were still clearly observed in light with an R:FR of 1, which allows shade avoidance. Despite
376 increased hyponasty in all lines in the interaction experiments carried out in these conditions,
377 possession of long period relative to the environmental light / dark cycle was associated with a
378 reduced ability to overtop the leaves of neighbors with a better-adapted diurnal leaf movement
379 rhythm.

380 Finally, although leaf overtopping was measured at a single time point, we observed that this
381 represents a fixed rather than changing outcome. The result of such interaction between two leaves is
382 determined over a relatively short period of time, early in the interaction as the two interacting leaves
383 meet. Beyond a certain point in the interaction, continued outward growth of the two interacting
384 leaves means that the final leaf position, on top or below, remains established, despite subsequent
385 leaf movements. Beyond that point leaf movements were insufficient to disrupt an established
386 overtopping outcome. This phenomenon is illustrated by the interaction shown in Supporting
387 Information Fig. S1.

388 These results demonstrate for the first time the existence of a potential overtopping advantage
389 associated with circadian clock-driven leaf movements. We present this as single clock regulated
390 mechanistic feature which may contribute to competition alongside other clock controlled processes
391 in plants such as regulation of diurnal starch usage patterns (Graf et al., 2010) and regulation of
392 flowering time (Song et al., 2015). We are keen to stress that this is quite distinct from a fitness
393 advantage such as that shown by Dodd et al. (2005). This overtopping advantage acts additively with
394 the overtopping advantage conferred by shade avoidance. Indeed, overtopping due to shade induced
395 hyponasty is, similarly, just one aspect of a wider phenomenon. It was many years after the initial
396 discovery of shade avoidance as a mechanism for overtopping of neighbors in competition for light
397 that an extensive analysis of the shade avoidance syndrome demonstrated that it does confer a
398 genuine fitness advantage in a natural environment (Schmidt 1997). Full demonstration of a concrete
399 fitness advantage for leaf movement as a mechanism of competition would require similar extensive

400 additional experimentation in natural conditions, measuring an output such as biomass or seed yield
401 and such a study is beyond the scope of this work. However, what we do show is that circadian
402 resonance of these leaf movements can confer a direct advantage in overtopping neighboring plants
403 in a competitive environment. We, therefore, add an additional possibility to those proposed by
404 Darwin in order to explain circadian leaf movements. We propose that these leaf movements may
405 form a mechanism that confers advantages to ground-level, rosette plants in competition for light.

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Figure Legends

Fig. 1. Wild type seedlings overtop long period mutant seedlings in 24 hour days. (A) Percentage of overlaps won by wild type (WT) and *zt1* mutant seedlings of Arabidopsis in direct competition in 24 hour days of 16 hours light / 8 hours dark. Bars show mean percentage of overlaps won by each genotype \pm 1SE for four replicate grids of seedlings. (B) Leaf angle measurements in wild type (WT) and *zt1* mutant seedlings grown in 24 hour days. Graphs show mean leaf angle for between 6 and 10 leaves \pm 1SE. Black bars represent dark periods.

Fig. 2. Phase of maximum leaf angle in wild type (WT) and long period mutant seedlings of Arabidopsis. Phase of maximum leaf angle in WT and *zt1* mutant seedlings in either 24 hour days of 16 hours light / 8 hours dark or in 27 hour days of 18 hours light / 9 hours dark. Graphs show mean peak phase represented as hours after dawn for individual daily peaks of leaf angle of between 6 and 10 leaves \pm 1SE. Mean phase of peak hyponasty in 27 hour days was corrected to represent the proportion of a standard 24 hour day / night cycle at which the peaks occurred. Asterisk represents a significant difference, $p=0.018$, based on a heteroscedastic t-test.

Fig. 3. Wild type and long period mutant seedlings compete equally in 27 hour days. (A) Percentage of overlaps won by wild type (WT) and *zt1* mutant seedlings of Arabidopsis in direct competition in 27 hour days of 18 hours light / 9 hours dark. Bars show mean percentage of overlaps won by each genotype \pm 1SE for four replicate grids of seedlings. (B) Leaf angle measurements in wild type (WT) and *zt1* mutant seedlings grown in 27 hour day conditions. Graphs show mean leaf angle for between 6 and 10 leaves \pm 1SE. Black bars represent dark periods.

Fig. 4. Short period mutant seedlings overtop wild type seedlings in 21 hour days. (A) Percentage of overlaps won by wild type (WT) and *toc1* mutant seedlings of Arabidopsis in direct competition in 21 hour days of 14 hours light / 7 hours dark. Bars show mean percentage of overlaps won by each genotype \pm 1SE for four replicate grids of seedlings. (B) Leaf angle measurements in wild type (WT)

and *toc1* mutant seedlings grown in 21 hour days conditions. Graphs show mean leaf angle for between 6 and 10 leaves \pm 1SE. Black bars represent dark periods.

Fig. 5. Phase of minimum leaf angle in wild type (WT) and short period mutant seedlings of Arabidopsis. Phase of minimum leaf angle in WT and *toc1* mutant seedlings of Arabidopsis in either 21 hour days of 14 hours light / 7 hours dark or in 24 hour days of 16 hours light / 8 hours dark. Graphs show mean trough phase for individual daily troughs of leaf angle of between 6 and 10 leaves \pm 1SE. Mean phase of peak hyponasty in 21 hour days was corrected to represent the proportion of a standard 24 hour day / night cycle at which the peaks occurred. Asterisk represents a significant difference, $p=0.011$, based on a heteroscedastic t-test.

Fig. 6. Wild type and short period mutant seedlings compete equally in 24 hour days. (A) Percentage of overlaps won by wild type (WT) and *toc1* mutant seedlings of Arabidopsis in direct competition in 24 hour days of 16 hours light / 8 hours dark. Bars show mean percentage of overlaps won by each genotype \pm 1SE for four replicate grids of seedling. (B) Leaf angle measurements in wild type (WT) and *toc1* mutant seedlings grown in 24 hour day conditions. Graphs show mean leaf angle for between 6 and 10 leaves \pm 1SE. Black bars represent dark periods.

Fig. 7. Arrhythmic mutant seedlings showing constitutive hyponasty overtop wild type seedlings. (A) Percentage of overlaps won by wild type (WT) and *CCA1* overexpressing (*CCA1ox*) seedlings of Arabidopsis in direct competition in 24 hour days of 16 hours light / 8 hours dark. Bars show mean percentage of overlaps won by each genotype \pm 1SE for four replicate grids of seedlings. (B) Leaf angle measurements in wild type (WT) and *CCA1ox* seedlings grown in the same 24 hour day conditions. Graphs show mean leaf angle for between 6 and 10 leaves \pm 1SE. Black bars represent dark periods.

Fig. 8. Wild type seedlings overtop long period mutant seedlings when seedlings are exhibiting shade avoidance responses. (A) Percentage of overlaps won by wild type (WT) and *ztl* mutant seedlings of Arabidopsis in direct competition in white light supplemented by far red light to give an R:FR of 1.0.

Seedlings were grown in 24 hour days of 16 hours light / 8 hours dark. Bars show mean percentage of overlaps won by each genotype \pm 1SE for four replicate grids of seedlings. (B) Leaf angle measurements in wild type (WT) versus *ztl* mutant seedlings grown in the same 16 / 8 days with R:FR of 1.0. Graphs show mean leaf angle for between 6 and 12 leaves \pm 1SE. Black bars represent dark periods.

Fig. 9. Phase of maximum/minimum leaf angle in wild type and period length mutant seedlings exhibiting shade avoidance responses. Arabidopsis seedlings were grown in white light supplemented by far red light to give an R:FR of 1.0, in 24 hour days of 16 hours light / 8 hours dark. (A) Phase of maximum leaf angle in wild type (WT) versus *ztl* mutant seedlings (B) Phase of minimum leaf angle in wild type (WT) versus *toc1* mutant seedlings. Graphs show mean peak phase for individual daily peaks of leaf angle of between 6 and 12 leaves \pm 1SE. Asterisk represents a significant difference, $p=1.20 \times 10^{-7}$, based on a heteroscedastic t-test.

Fig. 10. Wild type and short period mutant seedlings exhibiting shade avoidance responses compete equally in 24 hour days. (A) Percentage of overlaps won by wild type (WT) and *toc1* mutant seedlings of Arabidopsis in direct competition in white light supplemented by far red light to give an R:FR of 1.0. Seedlings were grown in 24 hour days of 16 hours light / 8 hours dark. Bars show mean percentage of overlaps won by each genotype \pm 1SE for four replicate grids of seedlings. (B) Leaf angle measurements in wild type (WT) versus *toc1* mutant seedlings grown in the same 16 / 8 days with R:FR of 1.0. Graphs show mean leaf angle for between 6 and 12 leaves \pm 1SE. Black bars represent dark periods.

Figure 1

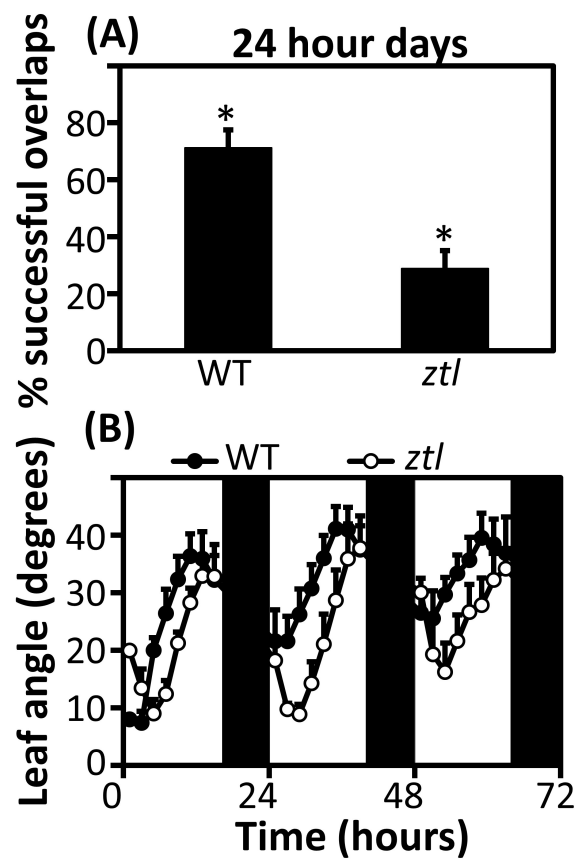


Figure 10

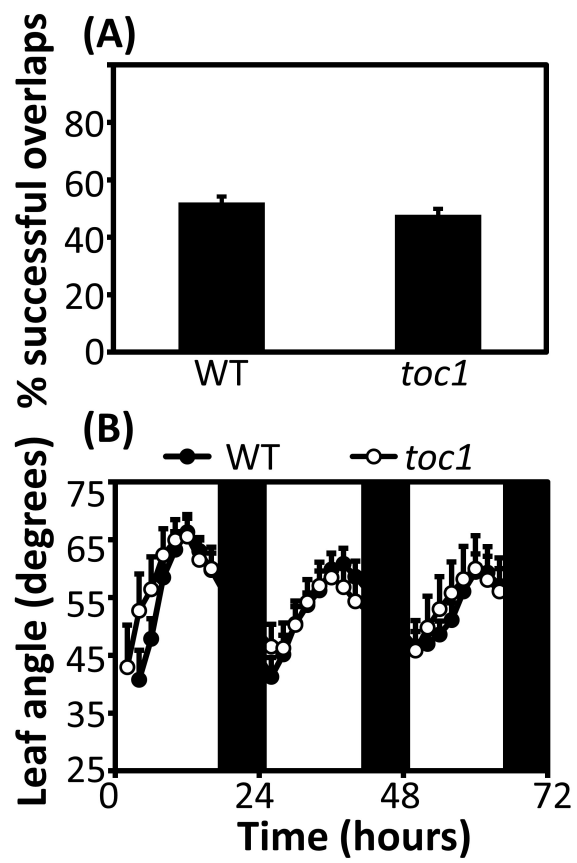


Figure 2

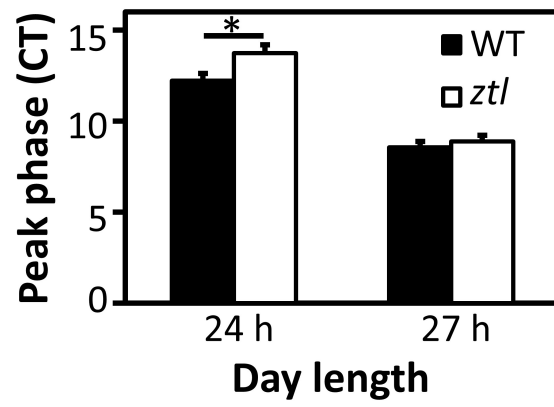


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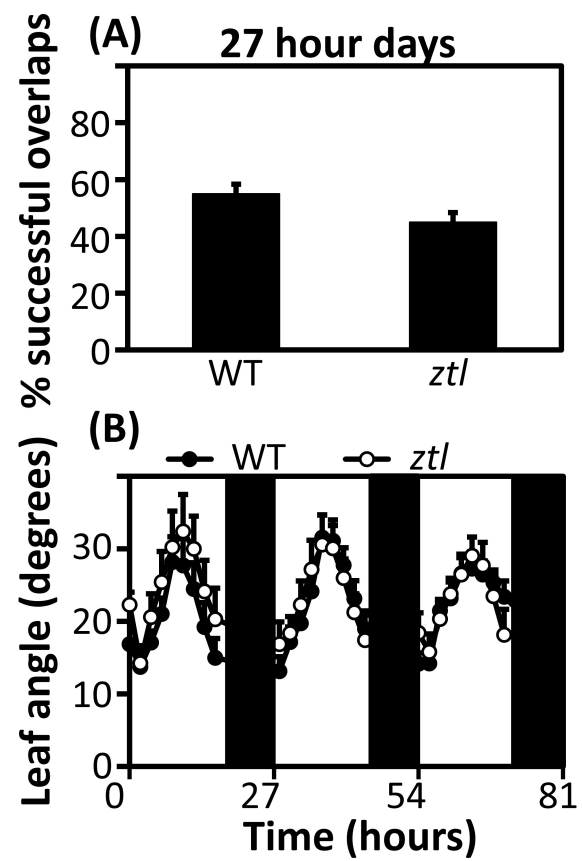


Figure 4

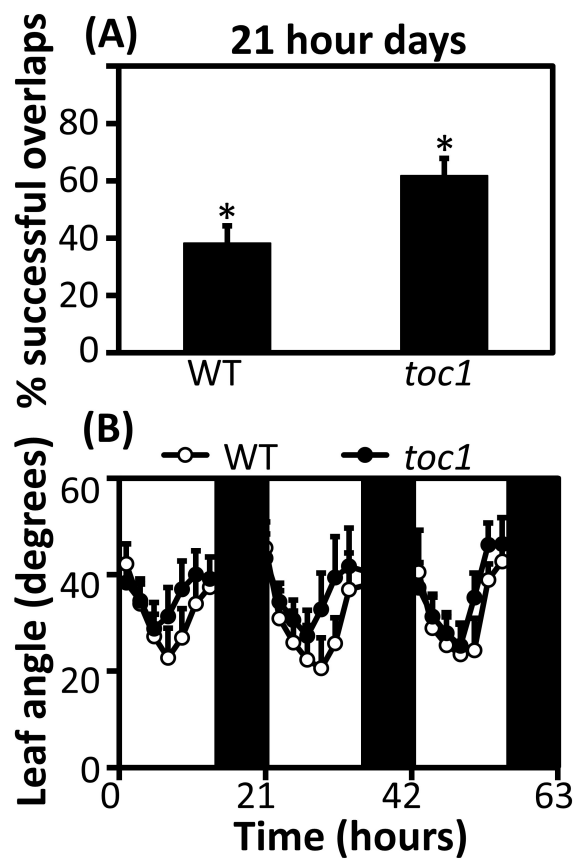


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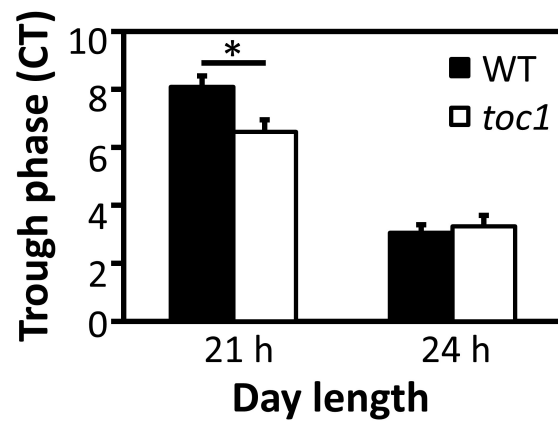


Figure 6

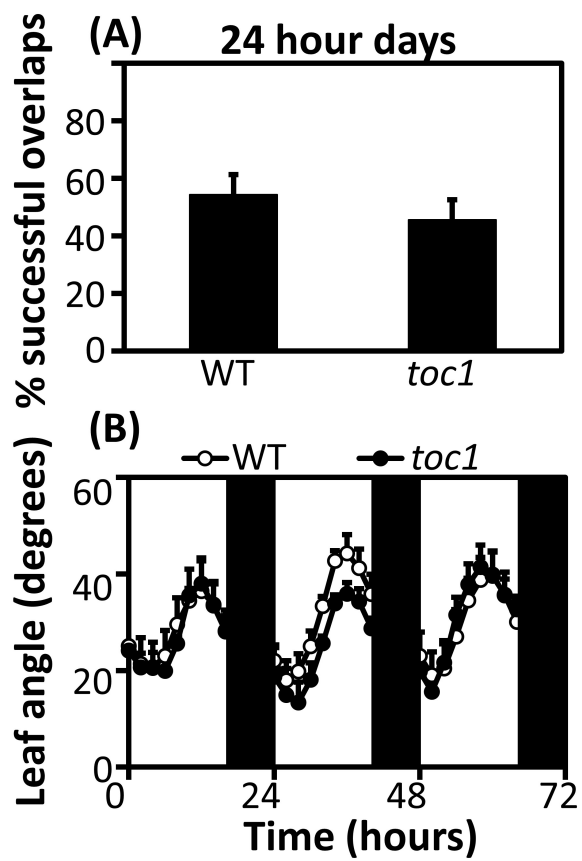


Figure 7

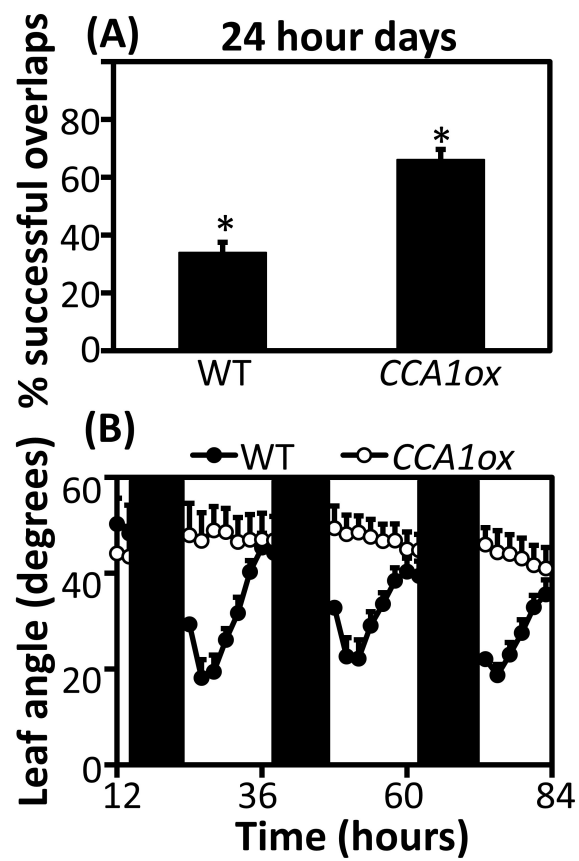


Figure 8

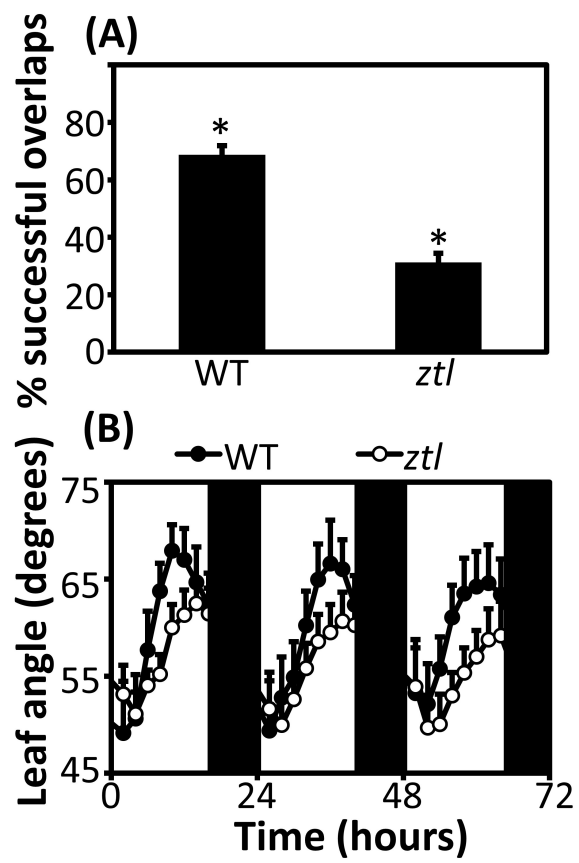


Figure 9

