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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.

Key Words
Circadian, hyponasty, leaf movement, overtopping
1. Introduction

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

Circadian leaf movements do not always involve the raising of leaves. Darwin observed a lowering
leaves to a vertical orientation at night in beans (Darwin, 1880). He was the first to suggest a
functional role for what he called the sleep movements of leaves, with his hypothesis that leaves held
vertically would radiate less heat at night than horizontally held leaves and so such movements might
protect plants from the effects of chilling or frost damage through the conservation of heat (Darwin,
1880). Experiments, which Darwin conducted on a number of plant species including *Trifolium* and
*Oxalis*, revealed that leaves forced into a horizontal position during periods of freezing temperature at
night suffered more frost damage than leaves that were allowed to assume a normal vertical
orientation (Darwin, 1880). However, Schwintzer (1971) suggested that Darwin’s findings were
probably artefactual after observing that vertical orientation of leaves at night failed to provide
adequate protection from freezing in soybeans as only small (<1°C) temperature differences were
revealed to exist between leaves held vertically and those held horizontally. Enright (1982) replicated
Schwintzer’s finding of only a small temperature difference between horizontally and vertically held
leaves, but also found that, under non-freezing conditions, warmer vertically oriented leaves grew
slightly more rapidly than horizontally oriented ones, indicative of a possible role for circadian leaf
movements as a protective mechanism against chilling as opposed to freezing in certain plants.
However, all such experiments investigated plants showing large changes in leaf orientation from horizontal during the day to near vertical orientation of leaves at night. These changes are the result of the action of specific organs called pulvini, particularly common in legumes, which raise or lower leaves as a result of changes in turgor pressure (Koller et al., 2000). In plants which lack pulvini leaf movements are simply the result of alternating abaxial and adaxial growth within the petiole as the leaf grows (Koller et al., 2000). Generally, this results in a much more subtle leaf movement. Nonetheless, this phenomenon has been demonstrated to result some frost protection in giant rosette plants such as *Senecio keniodendron* which inhabit tropical mountains. These plants hold their upper leaves at a very high angle during the day but raise them still further to form a closed “night-bud” during the night (Beck et al., 1982; Smith, 1974). In contrast, rosette plants such as dandelion or Arabidopsis which inhabit more temperate regions, raising their leaves from horizontal during the day to a maximum inclination no more than 45 degrees during the night (Mayer 1966; Millenaar et al., 2005). Such an occurrence would not seem likely to provide any degree of frost protection and the reason for such leaf movements is unexplained. We propose here that the circadian leaf movements in such rosette plants could have an additional benefit as a mechanism for facilitating overtopping of neighboring plants. These plants show a ruderal strategy and are generally early colonizers of open ground in spring, quickly producing seed (Pigliucci, 2002). Competition for light amongst such species is intense and such overtopping would be an advantage in preventing shading. In order to investigate this hypothesis, we chose the model plant, Arabidopsis. As mentioned, Arabidopsis features a characteristic rhythmic leaf movement that is believed to be based upon differential growth of the petioles (Engelmann and Johnsson, 1998). A circadian rhythm controls the elongation rates of abaxial and adaxial cells in turn in order to drive the positioning of leaves (Polko et al., 2012; Rauf et al., 2013). In Arabidopsis, as in other plants, leaves are raised during the day and fall again at the end of the night, a pattern of oscillation which continues following transfer to constant light (Hicks et al., 1996). High resolution, automated analysis of this phenomenon using near-infrared laser scanning has additionally revealed that leaf/petiole elongation in Arabidopsis also shows a circadian regulation of leaf elongation growth, with the peak of leaf/petiole elongation occurring in the morning just as leaves are rising (Dornbusch et al., 2014), further enhancing the proposition that leaf movement rhythms may be part of an overtopping mechanism. In our assay, we set up a series of experiments where wild type plants were placed in close proximity with a range of circadian clock mutants in a
range of day lengths. We were able to show that circadian clock-driven leaf movements in resonance with the external light/dark cycle do facilitate overtopping of neighboring plants which fail to show correct timing of these movements; thus, supporting our hypothesis. Furthermore, we show that this overtopping mechanism acts additively with overtopping due to shade avoidance.
2. Materials and Methods

2.1. Plant Materials and Growth Conditions

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

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

2.2. Proximity Experiments

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

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

3. Results

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

3.1. Interaction between wild type and a long period mutant

In our initial experiments, wild type plants were grown in direct proximity to the long period ztl long period mutant, which displays a 27 hour period (Somers et al., 2000). In light-dark cycles of 16 hours light / 8 hours dark, resonating with the wild type period length of 24 hours, leaves of wild type plants overtopped those of ztl in over 71% of cases on average (Fig. 1A) (p value, chi squared test, $7.38 \times 10^{-8}$ for a null hypothesis of no significant difference). Examination of leaf angles over a three day period
revealed that the leaf movement rhythms of both wild type and ztl showed a repeating 24 hour pattern. This is indicative of the action of light in entrainment of the normally long period ztl line to the external light / dark cycle. However, ztl leaf movement rhythms showed a consistent difference in phase relative to those of wild type seedlings suggesting an imperfect entrainment (Fig. 1B). Calculation of the mean phase of peak hyponasty, expressed as the time after dawn, revealed that wild type showed a mean peak phase at 12.22 h, while ztl showed a mean peak phase at 13.73 h (Fig. 2) (p value, heteroscedastic T-test, 0.018).

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

3.2. Interaction between wild type and a short period mutant

Wild type seedlings were then grown in direct proximity to a short period mutant, toc1, which displays an endogenous period of 21 hours (Strayer et al., 2000). When wild type seedlings were placed alongside toc1 in 21 hour days of 14 hours light / 7 hour dark cycles, we observed that toc1 showed a greater percentage of overlaps won than wild type (62% vs 38%; p value, chi squared test, 0.017 for a null hypothesis of no significant difference) (Fig. 4A). Once again, seedlings of the genotype adapting to a day length shorter than its endogenous period, showed an inability to properly entrain their leaf movements to these conditions. In this case, the wild-type seedlings adapted well to a 21 hour day whereas previously this same behavior was exhibited when the long period ztl mutant which had adapted well to a 24 hour day. Both wild type and toc1 seedlings showed
an apparent 21 hour repeating pattern length of leaf movement but wild type seedlings showed a delayed phase (Fig. 4B). For phase analysis in these 21 hour days, mean phase of leaf position was, again, corrected to represent the proportion of a standard 24 hour day / night cycle at which the peaks occurred. In this case mean trough phase was plotted as peak phase for some seedlings occurred at or beyond dusk. With leaf position beyond dusk being obscured due to absence of light beyond dusk, it was not possible to be certain of the exact peak phase for such seedlings. In this case, trough phase was plotted rather than peak phase as peak time was not always clear for every seedlings due to the peak being on the border of dusk, particularly for wild type seedlings. Mean trough phase for \textit{toc1} seedlings occurred at 6.53 h after dawn, while mean trough phase for wild type seedlings occurred at 8.09 h (Fig. 5) (p value, heteroscedastic T-test, 0.011). As previously, mean phase of peak hyponasty in this non 24-hour day was corrected to represent the proportion of a standard 24 hour day / night cycle at which the peaks occurred.

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

3.3. Interaction between wild type and an arrhythmic mutant

We then placed wild type plants in direct proximity to a well-characterized line overexpressing the \textit{CIRCADIAN CLOCK ASSOCIATED1} gene (\textit{CCA1ox}) that is part of the central clock loop (Hsu and Harmer). The \textit{CCA1ox} line lacks any circadian clock-driven leaf movements (Wang et al., 1998). To our surprise, in 24 hour days of 16 hours light / 8 hours dark, the \textit{CCA1ox} line showed significantly more overtopping of the opposing genotype than the wild type did (66% of overlaps won by \textit{CCA1ox} vs 34%
won by wild type) (p value, chi squared test, 0.003 for a null hypothesis of no significant difference)
(Fig. 7A). Analysis of leaf tip traces in these conditions showed that CCA1ox showed a constitutive extreme hyponasty (Fig. 7B), a feature shared with the only other unconditionally arrhythmic circadian clock mutant, the LATE ELONGATED HYPOCOTYL overexpressor, lhy-1 (Schaffer, 1997). Such extreme hyponasty may account for the observed overtopping of wild type seedlings. CCA1-ox did, however, show a minor environmentally-driven, non-anticipatory oscillation in leaf movement in these light / dark cycles with leaf angle showing a slight fall during the light period and a slight rise during the dark period (Fig. 7B) (leaf position begins each day slightly higher than it ended the previous day).

3.4. Interaction in white light supplemented by far red

Thus far, our interaction experiments were carried out in white light lacking any significant amounts of far red light (R:FR 5.1). Such conditions would greatly reduce the potential for the shade avoidance response that would normally form an additional part of interactions between neighboring plants in the wild. In natural light, depletion of red but not far red wavelengths in light reflected from neighboring plants results in a decreased R:FR and causes increased hyponasty as well as leaf and petiole elongation (Roig-Villanova and Martínez-García 2016) which might alter the importance of leaf movement as an overtopping strategy. In order to examine whether leaf movement rhythms also confer an overtopping advantage under more natural environmental conditions, we repeated the 24 hour day interaction experiments involving the ztl and toc1 mutants in white light supplemented with far red light, giving an R:FR of 1.0, similar to that of sunlight. Under these conditions, interaction between wild type and long period ztl mutant seedlings, again, resulted in a significant advantage to wild type seedlings in terms of overtopping events. In light-dark cycles of 16 hours light / 8 hours dark, leaves of wild type plants overtopped those of ztl in over 69% of cases on average (Fig. 8A) (p value, chi squared test, 1.55 x 10^{-7} for a null hypothesis of no significant difference). Analysis of leaf angle in these seedlings revealed that, at all time points tested, leaf angle was considerably greater under these conditions than in white light for both genotypes indicative of a marked shade avoidance response (Fig. 8B). Nonetheless, diurnal leaf movement rhythms were still clearly visible in both wild type and ztl mutant seedlings, demonstrating that the shade avoidance response did not obviate the leaf movement rhythm. As in white light, leaf movement rhythms of both wild type and ztl showed a
24 hour pattern but with ztl leaf movement rhythms showing a consistent difference in phase relative to those of wild type seedlings (Fig. 8B). Calculation of the mean phase of peak hyponasty revealed that wild type showed a mean peak phase at 12.08 after dawn, while ztl showed a mean peak phase at 14.58 h after dawn (Fig. 9A) (p value, heteroscedastic T-test, 1.20 x 10^{-7}). Thus, in light with a R:FR equivalent to sunlight, ztl seedlings also showed an inability to properly entrain their leaf movements to a day length shorter than their endogenous period and showed a reduced percentage of leaf overlaps compared to wild type.

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

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

4. Discussion

Darwin initiated research into the functional role of circadian clock-driven leaf movements over a century ago (Darwin, 1880) and this role has subsequently been the subject of much speculation. Here we illustrate a potential role for these movements in Arabidopsis as a mechanism of competition
for light. In the conditions of our study, we observed that circadian resonance of these leaf
movements can contribute to overtopping of the leaves of neighboring plants.

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

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

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

Another interesting observation was the occurrence of an earlier relative time of peak hyponasty in
longer day lengths. In 24 hour days versus 27 hour days (Fig. 2) or 21 hour days versus 24 hour days
(Fig. 5), all genotypes showed a relatively earlier peak leaf angle with respect to the total duration of the day when grown in the longer day length. This difference is more than can be accounted for by a simple determination of phase by dawn. Instead it appears that the preceding dusk may also play a significant role in determining the phase exhibited in the following day.

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

Surprisingly, the arrhythmic CCA1ox line gained an overtopping advantage over wild type plants in 24 hour light / dark cycles. It might be expected that the absence of leaf movements would be a significant disadvantage in terms of overtopping. However, our assays demonstrated a consistently high leaf angle in the arrhythmic line. Such a constitutive hyponasty would, no doubt, give a considerable advantage in overtopping neighbors. The fact that such extreme hyponasty has not evolved in wild type lines as an even better competitive strategy indicates that there must also be considerable negative trade-offs associated with it. It is likely that a constitutively high elevation angle will reduce light capture due to a reduced angle of incidence to the sun. These negative trade-offs may, indeed, be part of the reason why CCA1ox plants show reduced net carbon fixation and, ultimately, reduced aerial biomass when grown in monoculture in these same conditions (Dodd et al., 2005). However, this issue does raise the issue of the wider implications of leaf movements.
may be other positive or negative effects on light capture for example which we do not examine here. We merely examine one aspect here, overtopping advantage, which will likely be part of a much larger balanced equation of positive and negative impacts in the real environment in the ultimate determinant of fitness.

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

Another aspect of the controlled environment nature of our study is the fact that our experimental grid had an edge. Edge effects have been observed in a number of ecological situations and can result in competitive advantages not being equally realized by all populations at the edge of a habitat. We did not expect wild type and period length mutants to be differentially affected at the outer edge of the grid versus the middle of the grid and, indeed, a calculation of overlaps for plants at the outer edge versus the middle showed no difference in percentage competition won by each genotype.
among plants at the edge of the grid. For example, wild type versus toc1 in 24 hour days wild type leaves won 50.5% of interactions, toc1 49.5% at the edges of the grid, almost identical to the pattern seen for the grids as a whole in this assay.

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

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

These results demonstrate for the first time the existence of a potential overtopping advantage associated with circadian clock-driven leaf movements. We present this as single clock regulated mechanistic feature which may contribute to competition alongside other clock controlled processes in plants such as regulation of diurnal starch usage patterns (Graf et al., 2010) and regulation of flowering time (Song et al., 2015). We are keen to stress that this is quite distinct from a fitness advantage such as that shown by Dodd et al. (2005). This overtopping advantage acts additively with the overtopping advantage conferred by shade avoidance. Indeed, overtopping due to shade induced hyponasty is, similarly, just one aspect of a wider phenomenon. It was many years after the initial discovery of shade avoidance as a mechanism for overtopping of neighbors in competition for light that an extensive analysis of the shade avoidance syndrome demonstrated that it does confer a genuine fitness advantage in a natural environment (Schmidt 1997). Full demonstration of a concrete fitness advantage for leaf movement as a mechanism of competition would require similar extensive
additional experimentation in natural conditions, measuring an output such as biomass or seed yield
and such a study is beyond the scope of this work. However, what we do show is that circadian
resonance of these leaf movements can confer a direct advantage in overtopping neighboring plants
in a competitive environment. We, therefore, add an additional possibility to those proposed by
Darwin in order to explain circadian leaf movements. We propose that these leaf movements may
form a mechanism that confers advantages to ground-level, rosette plants in competition for light.

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References


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 ztl 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 ± 1SE for four replicate grids of seedlings. (B) Leaf angle measurements in wild type (WT) and ztl mutant seedlings grown in 24 hour days. Graphs show mean leaf angle for between 6 and 10 leaves ± 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 ztl 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 ± 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 ztl 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 ± 1SE for four replicate grids of seedlings. (B) Leaf angle measurements in wild type (WT) and ztl mutant seedlings grown in 27 hour day conditions. Graphs show mean leaf angle for between 6 and 10 leaves ± 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 ± 1SE for four replicate grids of seedlings. (B) Leaf angle measurements in wild type (WT)
and \textit{toc1} mutant seedlings grown in 21 hour days conditions. Graphs show mean leaf angle for between 6 and 10 leaves ± 1SE. Black bars represent dark periods.

\textbf{Fig. 5.} Phase of minimum leaf angle in wild type (WT) and short period mutant seedlings of \textit{Arabidopsis}. Phase of minimum leaf angle in WT and \textit{toc1} mutant seedlings of \textit{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 ± 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.

\textbf{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 \textit{toc1} mutant seedlings of \textit{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 ± 1SE for four replicate grids of seedling. (B) Leaf angle measurements in wild type (WT) and \textit{toc1} mutant seedlings grown in 24 hour day conditions. Graphs show mean leaf angle for between 6 and 10 leaves ± 1SE. Black bars represent dark periods.

\textbf{Fig. 7.} Arrhythmic mutant seedlings showing constitutive hyponasty overtop wild type seedlings. (A) Percentage of overlaps won by wild type (WT) and \textit{CCA1} overexpressing (\textit{CCA1ox}) seedlings of \textit{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 ± 1SE for four replicate grids of seedlings. (B) Leaf angle measurements in wild type (WT) and \textit{CCA1ox} seedlings grown in the same 24 hour day conditions. Graphs show mean leaf angle for between 6 and 10 leaves ± 1SE. Black bars represent dark periods.

\textbf{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 \textit{ztl} mutant seedlings of \textit{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 ± 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 ± 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 ± 1SE. Asterisk represents a significant difference, p=1.20 x 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 ± 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 ± 1SE. Black bars represent dark periods.
Figure 1

(A) 24 hour days

Leaf angle (degrees) % successful overlaps

(B) Time (hours)

WT vs ztl
Figure 10

(A) Bar chart showing the percentage of successful overlaps between WT and toc1 genotypes. The bars indicate that WT has a higher percentage of successful overlaps compared to toc1.

(B) Line graph showing the leaf angle (degrees) over time (hours) for WT and toc1 genotypes. The graph shows a periodic pattern with a peak at around 24 hours and a trough at around 48 hours, with WT maintaining a higher leaf angle compared to toc1.
Figure 2

![Graph showing peak phase (CT) over day length](Image)
Figure 3

(A) 27 hour days

(B) Leaf angle (degrees) vs. % successful overlaps

WT  ztl

Time (hours) 0 27 54 81
Figure 4
Figure 5

![Graph showing Trough phase (CT) vs Day length with two conditions: WT and toc1. The graph indicates a significant difference (*p* < 0.05) between the two conditions at 21 h.]
Figure 6

(A) 24 hour days

(B) Leaf angle (degrees) vs. % successful overlaps

WT vs. toc1

Time (hours)
Figure 7

(A) 24 hour days

Leaf angle (degrees) % successful overlaps

WT CCA1ox

(B) Time (hours)

Leaf angle (degrees)
Figure 8

(A) Leaf angle (degrees) vs. % successful overlaps.

(B) Leaf angle (degrees) over time (hours) for WT and ztl.
Figure 9

(A) Peak phase (CT) for WT and ztl genotypes. 
(B) Peak phase (CT) for WT and toc1 genotypes.