Effect of Different Photoperiods on Flower Opening Time in *Portulaca umbraticola*

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*Portulaca umbraticola* is an ephemeral flower that opens early in the morning and wilts in the late afternoon. Although light and temperature act as major external cues to limit the velocity of flower opening, endogenous factors regulating its timing are largely unknown. In this study, we used time lapse photography to study the effect of different photoperiods and light qualities on the flower opening rhythm of *Portulaca umbraticola*. When illumination was provided, flower opening was rapid and most of the flowers reached the full opening stage. In contrast, in continuous darkness (DD), progression of flower opening was similar to other treatments only during the earlier stages of flower opening; thereafter, progression was significantly slower and most flowers did not progress up to the full opening stage. A robust flower opening rhythm with a period of approximately 24 h was observed in DD for at least three days and flower opening was strongly synchronous. In contrast, continuous white (LL) and continuous red (RR) lights showed a less robust rhythm with periods of approximately 21 and 22 h, respectively, for the first two days and from the second to the third day arrhythmia developed. Continuous blue light (BB) mirrored DD, with a period of approximately 25 h. Under the different photoperiods used (20L/4D, 18L/6D, 16L/8D, 12L/12D, 8L/16D, and 4L/20D), flower opening occurred earlier at longer photoperiods in comparison with shorter photoperiods, relative to the reference point (17:00). However, when the dark period was less than 6 h, loss of synchronicity of flower opening was observed. Synchronicity of flower opening was only set when the dark period was greater than or equal to 6 h.

Key Words: circadian rhythm, ephemeral, light-dark cycles, synchronous.

Introduction

Flower opening and closure are closely associated with successful reproduction, as they allows pollen removal and/or pollination (van Doorn and van Meeteren, 2003). Flower opening is categorised into different forms such as diurnal (day-bloomer; example *Empyreuma pugione* flower) and nocturnal (night-bloomer; example *Oenothera biennis*). Within diurnal and nocturnal flowers, repetitive and single opening and closing patterns can be observed. Flowers of *Silene saxifraga* showed repeated opening and closure during the night and day, respectively (Halket, 1931). Some flowers such as the ephemeral *Hibiscus rosa-sinensis* L. open and close once, with a vase life of 12–18 h (Trivellini et al., 2007). Environmental factors play a fundamental role in flower opening and closure. Light has been shown to play an important role in the opening of many flowers, particularly diurnal ones with relative humidity being important for nocturnal species.

Some day-bloomer species and night-bloomer species have been shown to have an endogenous rhythm of flower opening and closure (van Doorn and van Meeteren, 2003). In recent studies, the involvement of a circadian rhythm has been shown in the opening of *Eustoma grandiflorum*, as well as in petals of cut roses (Bai and Kawabata, 2015; Horibe and Yamada, 2014). The phenotypic rhythms displayed by plants depends upon a complex interplay of interacting endogenous rhythmic controls and environmental signals (Millar, 2004). Circadian clocks in plants are known to be entrained by light and temperature signals from the environment. Thus, tampering with these inputs could result in entrainment of the clock, thereby modifying the phenotypic rhythms displayed by the plants. Recent molecular-genetic studies in the model plant *Arabidopsis* have revealed that the circadian system is composed of an input pathway, central oscillator, and...
output pathway. The light signals perceived by photoreceptors such as red/far-red receptor phytochromes and blue/UV-A receptor cryptochromes entrain the clock. The central oscillator is composed of multiple interlocked feedback loops that include CIRCADIAN CLOCK-ASSOCIATED 1 (CCA1), LATE ELONGATED HYPOCOTYL (LHY), TIMING OF CAB EXPRESSION 1 (TOC1), PSEUDO RESPONSE REGULATORS (PRR5/7/9), EARLY FLOWERING 3 (ELF3), GIGANTEA (GI), and ZEITLUPE (ZTL) (Greenham and McClung, 2015; Hsu and Harmer, 2014). The clock regulates specific outputs such as PHOTOCROME INTERACTING FACTORS (PIFs) or CONSTANS (CO) to control photoperiodic growth and flowering (Huang and Nusinow, 2016).

Portulaca umbraticola is an ephemeral flower that opens in the morning and withers in the late afternoon. In P. umbraticola ‘Single Red’ and ‘Sanchuraka Cherry Red’, it has been shown that a rise in temperature significantly affects opening (Maguvu et al., 2016). Ichimura and Suto (1998) reported that the flowers of Portulaca do not fully open on cloudy or relatively cool days. However, general observations have shown that in P. umbraticola, at a constant temperature, flowers of the same cultivar show some variations in flower opening time depending on the season, as well as weather conditions. The flowers open at an earlier time in summer compared to other seasons, so day length probably has some significant effects on the rhythm of flower opening and closure of P. umbraticola.

In this research, we concentrated on the effect of different photoperiods on the time of flower opening. The plants were also subjected to continuous illumination under different light sources, and the effect of continuous darkness was also assessed. The main objectives being 1.) To assess the effect of different photoperiods on flower opening time in P. umbraticola. 2.) To confirm the participation of a circadian clock in P. umbraticola flower opening. 3.) To assess the effect of different light qualities on the rhythm of flower opening.

Materials and Methods

Plant materials

P. umbraticola ‘Single Red’ (SR) was used as the experimental material in all experiments. This cultivar is almost sterile, and was vegetatively propagated by means of cuttings. The plants were grown in 15 cm plastic pots filled with a 3:1 mixture of granular soil and peat-based soil mix (Metro-Mix 360; Sun Gro, Agawam, MA, USA). These potted plants were raised at The University of Tokyo, in a phytotron at 28/23°C day and night temperatures, respectively, under natural sunlight. Potted plants bearing many flower buds (Fig. 1A) were used in these experiments. The number of flower buds to open on a particular day was not constant, but at least 3 buds opened every day. The plants were taken directly from the phytotron, and placed in a dark room, which was maintained at 25 ± 2°C and a relative humidity of about 20–40%.

Data collection and analysis

From the onset of each treatment, time lapse photographs were taken at 15 or 30 mins intervals using a digital camera (Optio WG-2; Ricoh Imaging, Tokyo, Japan). The flush function was used in darkness. Images were then displayed on a computer screen and the stages of flower opening were assigned through comparison with the stages shown in Figure 1B. Stage 2 of flower opening was used as the reference point (flower opening stage for this experiment); 3. Petal unfolding begins, and the petals are still attached to each other at the top; 4. Unfolding progresses as petals clearly separate at the top; 5. Fully open flower.
ments started at 17:00 Japan Standard Time (JST). It takes a single cycle for an inverted day-night cycle to invert the time of flower opening in \( P. \text{umbraticola} \) (data not shown). Therefore, in these experiments the first day of the treatments was not included in the results to avoid the effects of transition. For four consecutive days, only data from the second day onwards was used. Experiments were conducted from October–December 2015 as well as April–August 2016, and each experiment was replicated at least three times on different days using different pots for each replication.

Experiment 1. Effect of light exposure on flower opening

\( P. \text{umbraticola} \) buds were harvested directly from the phytotron at stage 1 (see Fig. 1B) and immediately placed in test tubes containing distilled water and then placed in continuous darkness (DD) and continuous white (LL) (under the above conditions). The degree of opening was assessed by comparison with stages shown in Figure 1B. For flower closure, the flowers were assigned stage numbers (7, 8, 9, 10, and 11), these stages were analogous to opening stages (5–1), respectively (see insert on Fig. 2). For construction of the graph, development stages which were in-between the designated stages were determined in relative terms. A single bud was used for each treatment and the experiment was replicated three times.

Experiment 2. Flower opening rhythms under continuous darkness (DD), white light (LL), red light (RR), and blue light (BB)

The potted plants were transferred into a dark room at the beginning of the treatments. Except for the camera flash, no other form of lighting was provided in DD so the plants were in complete darkness for four consecutive days. To assess the effects of continuous illumination, the above procedure was followed, the only difference being that for LL, a white fluorescent light (15–25 μmol·m\(^{-2}\)·s\(^{-1}\), FPL27EX-N; Matsushita Denko, Osaka, Japan) was used. For monochromatic red or blue light exposure, red (15–25 μmol·m\(^{-2}\)·s\(^{-1}\), 650–670 nm; OSR7CA5111A; OptoSupply, Hong Kong, China) or blue (15–25 μmol·m\(^{-2}\)·s\(^{-1}\), 465–475 nm; OSB56A5111A; OptoSupply) lights were used.

Experiment 3. Effect of different photoperiods on the time of flower opening

The potted plants bearing many flower buds were subjected to different photoperiods under a 24 h time cycle. Lighting was provided by a white fluorescent light as above and for darkness, the lights were turned off. The plants were treated to the following photoperiods (20L/4D, 18L/6D, 16L/8D, 12L/12D, 8L/16D, and 4L/20D) for four consecutive days and the images were captured as described above. For basic reference, all the night treatments started at 17:00 (JST).

Results

Differences in flower opening process under continuous light or darkness

Flower opening in \( P. \text{umbraticola} \) is a rapid process which when the conditions are optimal, takes less than 6 h from the bud stage (stage 1) up to full opening (stage 6). At 25 ± 2°C, opening proceeded gradually from the bud stage (stage 1) up to stage 2 (flower opening) in both DD and LL (Fig. 2), there were no clear differences between the two conditions (DD and LL). However, as the degree of opening progressed, opening was rapid in LL, it took about 6 h for the flower to progress up to stage 6 via stages 3, 4, and 5. In contrast, in DD, opening progressed slowly from stage 2 up to stage 5 via stage 3 and 4. It took about 12 h for plants in DD to progress up to stage 5 from stage 2 (Fig. 2). Although flowers in DD progressed past stage 5, most of them could not reach stage 6. Under the conditions used in these experiments, light was essential for full and rapid flower opening (Figs. 2 and 3). The flowers in LL closed about 10 h earlier than those in DD (Fig. 2).

1. Effects of continuous darkness and continuous illumination on flower opening rhythm

To assess whether the time of flower opening is regulated by an endogenous rhythm in \( P. \text{umbraticola} \), flower opening was examined under constant conditions. Potted plants bearing many flower buds were transferred to a dark room and the flower opening process was recorded for 4 consecutive days under constant conditions with or without illumination. In \( \text{Portulaca} \), each flower opens only once, and it wilts within a single day. In DD, a robust rhythm with a period of approximately 24 h was observed for at least 3 days, and
the opening time of each flower was strongly synchronous (Fig. 3A). This synchronicity persisted throughout the three days even under the absence of external light/dark cues. In addition, the flowers opened at subjective dawn throughout the experiment. The progression of flower opening from stage 2 up to stage 6 was very slow and in most cases the flowers could not reach stage 6 (Figs. 3C and 2). Therefore, light is essential for rapid and full opening. In contrast, in LL, circadian rhythm seemed to be conditionally sustained. A less robust rhythm with a period of approximately 21 h was observed during the first two days, which resulted in arrhythmia on the third day (Fig. 3B Replication 1). From the first to the second day, individual flower opening was synchronous; however, from the second to the third day the synchronicity was lost. Moreover, the circadian time (CT) of flower opening varied on all three days; on the second day flowers opened about 3 h earlier than the first day. In contrast, on the third day of flower opening, the flowers showed batches of opening which had a range of about 2.5–14 h earlier than the second day (Fig. 3B). Replication 2 and 3 showed a tendency similar to replication 1 during the first two days; however, from the second to the third day they showed less synchronicity (complete arrhythmia) of flower opening than replication 1. The progression from stage 2 to stage 6 was rapid in comparison with DD and in most cases all the flowers reached stage 6 (Figs. 3D and 2). The discrepancies observed in LL replications are probably due to differences in photoperiods between the seasons in which experiments were conducted, as well as slight differences in weather conditions on the day before transfer to the controlled room.

To assess the effect of different light quality on the rhythm of flower opening, the flower opening process was examined under continuous monochromatic red (RR) or blue (BB) light. In RR, a less robust rhythm with a period of approximately 22 h was observed for the first two days, resulting in arrhythmia on the third day. Synchronicity of individual flower opening mirrored that of LL (Fig. 4A). Also, the flowers opened at different CT on all days, with flowers opening 2 h earlier on the second day compared to the first day. On the third day, batches of flower opening were observed 3–14 h earlier than the second day (Fig. 4A). The flowers in RR also rapidly progressed to stage 6 just as in LL (Fig. 4C). The rhythm in RR was almost the same as that in LL (Figs. 4A and 3B). BB showed a robust rhythm of flower opening with an oscillation period of about 25 h (Fig. 4B). Individual flower opening was synchronous; however, unlike in DD progression, from stage 2 up to 6 of flower opening was moderately fast. It was faster than DD but slower than RR and LL. Most of the flowers managed to reach stage 6 of flower opening (Fig. 4D). The flowers opened on almost the same CT on all three days.

2. Effects of different photoperiods on the time of flower opening

The potted plants with many flower buds were sub-
jected to different photoperiods (20L/4D, 18L/6D, 16L/8D, 12L/12D, 8L/16D, and 4L/20D) for 4 consecutive days, and the time of flower opening was recorded from the second day onwards. All the dark treatments started at 17:00 (JST) in order to have a basic reference point. Flowers opened earlier with longer photoperiods than with shorter photoperiods relative to the reference point (17:00). However, when the dark period was less than 6 h, there was no synchronicity of flower opening (Fig. 5). At least 6 h darkness was required to maintain synchronicity of flower opening and when the dark period was greater than or equal to 6 h flowers opened at the same time every day. Flowers opened at approximately 23:30–00:00, 01:00, 03:30–04:00, 04:30–05:00, and 05:30–06:00 in 18L/6D, 16L/8D, 12L/12D, 8L/16D, and 4L/20D photoperiods, respectively (Fig. 5). In 20L/4D, there was no synchronicity of flower opening and the time of flower opening was different throughout the 3 recorded days. The speed of flower opening was not greatly affected in different photoperiods. However, the speed was moderately fast in all the photoperiods except for 4L/20D and 8L/16D because in all the other photoperiods, flowers reached the opening stage (stage 2) when the transition from dark to light was about to occur, but for 4L/20D and 8L/16D, it was still in darkness (Fig. 5).

Discussion

A hallmark of circadian regulation is the persistence of robust, accurate rhythms for many days under conditions of LL or DD (Doyle et al., 2002). Under these controlled conditions, the organism is deprived of external time cues, and a free running period of approximately 24 h is observed (McClung, 2006). In DD, flower opening showed a robust rhythm with a period of approximately 24 h (Fig. 3A), confirming the participation of the circadian clock in regulation of *P. umbraticola ‘SR’* flower opening. Flower opening was strongly synchronous and the flowers opened at the same CT (subjective dawn) throughout the recorded period. In contrast, in LL, flower opening showed a less robust rhythm with a period of approximately 21 h for at least two cycles and thereafter developed arrhythmia; the flowers opened at different CT for all three consecutive days (Fig. 3B). Thus, the circadian oscillation of flower opening in *P. umbraticola* was maintained in DD better than in LL. In *Eustoma grandiflorum* flowers, a rhythm with a period of approximately 25 h was observed for at least three days under constant darkness, indicating the involvement of the circadian clock (Bai and Kawabata, 2015). Moreover, this rhythm was also observed in constant red and blue lights. However, *Eustoma* flowers were arrhythmic in continuous white light showing no oscillations. In contrast, *Portulaca* showed a robust rhythm in continuous darkness as well as in blue light, but arrhythmia was observed under RR and LL. These differences are common, and as there are many reports which show persistence of these rhythms under different conditions, this may be species-specific. Flowers of *Kalanchoe blossfeldiana* showed circadian rhythm under both LL and DD (Karve et al., 1961), while those of *Bellis perennis* showed circadian rhythms under LL. The robust rhythm in DD and arrhythmia in LL suggests that clock output genes that

![Fig. 4. Flower opening rhythm under RR (A), BB (B), progression of flower opening in RR (C) and BB (D). (A) and (B) represents stage 2 while (C) and (D) represents stage 6. White and grey bars represent subjective day and night periods, respectively. The experiment was replicated three times on three different days using different potted plants. Individual experimental data are shown.](https://example.com/fig4.png)
regulate the rhythm of flower opening are activated in darkness. This was also suggested for the expression rhythm of floral inducer genes in Pharbitis nil (PnFT1 and PnFT2), which showed arrhythmia in LL but had robust rhythms in DD (Hayama et al., 2007).

The free-running period length of the clock is closely tied to light intensity. Aschoff’s rule states that, as light intensity decreases the period length of the rhythm lengthens in diurnal organisms and shortens in nocturnal organisms. This is presumed to be the net effect of decreased input to the clock by the resetting photoreceptors (Aschoff, 1979). In the P. umbraticola ‘SR’, the free running period was clearly longer in DD than in LL and RR (Figs. 3A, B, and 4A), and this was expected as it obeys Aschoff’s rule. However, the difference was not clear between DD and BB, suggesting that phytochrome is the primary photoreceptor mediating light input to the clock in the control of flower opening rhythm of P. umbraticola. In LL and RR P. umbraticola showed variable period length and impaired circadian rhythms (Figs. 3B and 4A), which is similar to the arrhythmic phenotype of the early flowering 3 (elf3) mutant in Arabidopsis (Hicks et al., 1996). In wild type Arabidopsis plants, ELF3 acts in the core clock component and sustains rhythmicity in long photoperiods and in LL by inhibiting phototransduction (gating) at a particular time of day (Covington et al., 2001). However, though our results have some degree of similarities with elf3 mutants, the circadian clock is a complex system and variations can be found from species to species, so the reason for the variable period length in LL in P. umbraticola will remains unclear until there is further molecular evidence. Recent studies in Nicotiana attenuata have reported that silencing of clock component genes NaLHY and NaZTL resulted in altered circadian rhythms of flower opening, floral scent emission, and vertical movement of flowers (Yon et al., 2016). This report clearly demonstrated the involvement of the circadian clock components in the rhythmic regulation of physiological processes in floral organs. Identification and functional analyses of clock-related genes in P. umbraticola would greatly advance our understanding of how clock components regulate one of the specific outputs such as the timing of flower opening.

In the conditions used in this experiment, light proved to be extremely influential on the speed and
degree of opening. Flower opening from stage 2 up to stage 6 progressed faster in LL than in DD (Figs. 2, 3C, and D). Moreover, most of the flowers in DD could not reach stage 6 of flower opening. These results augment the findings of Ichimura and Suto (1998) in which light intensified the response of flower opening in *Portulaca*, with flowers reaching full open stage 1 h after illumination. Partial opening of flowers in darkness has been reported in many flowers such as *Eustoma grandiflorum* and the Asiatic lily (Bai and Kawabata, 2015; Bielecki et al., 2000). In the Asiatic lily when the flowers were held in extended darkness, petals opened to ~40°, and anthers remained intact. In addition, the Asiatic lily also showed synchronous flower opening; however, both LL and DD resulted in loss of synchronicity. In contrast, in *P. umbraticola*, synchronicity was only lost in LL or when the dark period was less than 6 h (Figs. 5 and 3B). This shows that the effects of light and darkness on flower opening vary from species to species.

Taking (16L/8D) to be the standard cycle during summer under the different photoperiods used (20L/4D, 18L/6D, 16L/8D, 12L/12D, 8L/16D, and 4L/20D), increasing the photoperiod resulted in an earlier time of opening compared to the standard cycle, relative to the reference point (Fig. 5). In contrast, at photoperiods which were shorter than the standard cycle (12L/12D, 8L/16D, and 4L/20D), flower opening was delayed in comparison to the standard cycle, there was no proper correlation between photoperiods and flower opening time, but advances and delays could be observed. More interestingly, at (20L/4D), although flower opening was advanced, there was no synchronicity of flower opening (Fig. 5) just like in LL (Fig. 3B). After this observation, we experimented with different photoperiods and we found that there was a minimum period of darkness required for synchronicity in flower opening. Synchronicity occurred when the dark period was greater than or equal to 6 h. Thus, both light and darkness are necessary for setting the time of flower opening. Loss of synchronicity in flower opening time at longer photoperiods and LL is not unique, and the periods and amplitude of circadian rhythm are known to change without a zeitgeber, such as changes in light to darkness or vice versa (Jones and Mansfield, 1975). The ELF3 of *Arabidopsis* gates light input to the clock maintaining oscillations in LL, and the elf3 mutant showed conditional arrhythmia in LL. McWatters et al. (2000) demonstrating that this conditional arrhythmia masks an underlying oscillator function which can be revealed in DD. The oscillator will be arrested or become dysfunctional after the first subjective day in LL. Taking this into account, the arrhythmia in 20L/4D and LL suggests that the *P. umbraticola* ELF3 may have a weaker function specifically in the floral organs. The *P. umbraticola* ELF3 may easily be arrested or become dysfunctional in longer light periods or in LL. Molecular evidence is required to clarify this issue.

Increasing the photoperiod resulted in an earlier flower opening time relative to the reference point. This probably explains the observed differences in flower opening time between summer and other seasons as in summer the photoperiods are longer. In results comparable to ours, lengthening the night (8, 12, and 16 h) and shortening the day in the Asiatic lily delayed flower opening (Bielecki et al., 2000). In other words lengthening the day advanced the time of flower opening. In *Arabidopsis*, the phase of CHLOROPHYLL a/b BINDING PROTEIN (CAB) expression is predominantly set by the dark-to-light transition at dawn. However, in the elf3 mutant, the circadian clock is arrested during the light period and the phase of oscillation can be set by the light-to-dark transition (McWatters et al., 2000). Similarly in *P. nil* PnCAB expression under DD shifted gradually as the duration of the time spent in light increased suggesting that PnCAB expression is strongly influenced by the timing of the transition from dark-to-light at dawn or the duration of the time spent in light (Hayama et al., 2007). Kaihara and Takimoto (1979) reported that flower opening of *P. nil* occurred at a constant time after dusk (10 h) regardless of the entrained period when the light period was longer than 10 h. Thus, flower opening of *P. nil* is regulated by an endogenous circadian rhythm set by dusk and the absolute duration of darkness is a major determinant to opening time. Unlike in *P. nil*, the timing of flower opening in *P. umbraticola* is not determined simply by duration of darkness, but is also affected by day length (Fig. 5).

Taking into account these observations, changes in the flower opening time under different photoperiods and the arrhythmia when the dark period was less than 6 h in *P. umbraticola* suggest that both the dusk signal and duration of light period significantly influence the phase at which flower opening occurs. Also, in an analogous scenario in *Phaseolus vulgaris* L, stomatal opening in photoperiods which were shorter than 12 h plants showed a 1:1 relationship between the delay in the time of light-on and the delay in the phase of stomatal opening (Holmes and Klein, 1986).

In an interesting practical application, inverting day to night time resulted in a 12 h shift in the time of flower opening (data not shown), this occurred after only one cycle. Based on these observations we also subjected the plants to different cycles starting at different times and recognised that we could control the time of flower opening across a 24 h period (data not shown). Since *P. umbraticola* is an ephemeral flower which lasts only for a few hours, we can regulate flower opening to our desired time.

**Conclusion**

A circadian rhythm with a period of approximately 24 h was involved in *P. umbraticola* ‘SR’ flower opening. This was observed most prominently under continuous darkness. Light played a pivotal role in
determining the speed and extent of flower opening. However, the timing of flower opening was regulated by endogenous circadian rhythms in which dark to light transitions were essential for determining the phase of the rhythm, and a dark period of 6 h or more was essential to maintain synchronicity in the flower opening time. The differences in flower opening time between summer and other seasons were due to the difference in the length of the photoperiods (Fig. 6).

**Literature Cited**


