博士論文（要約）

Studies on the diversity of *Portulaca umbraticola* cultivars

（ハナスベリヒユ園芸品種の多様性に関する研究）

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CHAPTER 1

Introduction

1.1 Genus *Portulaca*

Portulaca plants belongs to the family Portulacaceae and genus *Portulaca* having over forty species. The plants are mostly annual, but herbaceous perennials also exist. They are distributed worldwide mainly in America, Africa, Asia, and Australia with some slight variations observed in different dimes of the same species. They mainly consist of flesh leaves which may be alternate, sub-alternate or opposite having conspicuous or inconspicuous hairs in the axils; this can be variable (having some slight modifications) depending on species and geographical location. They consist of purplish-red to green stems which are mostly crippling. They consist of two sepals which are fused at the base of the ovary. The sepals and petals of the family Portulacaeae are of bracteal origin, they are distinct from floral parts of other dicotyledonous plants (Ronse De Craene, 2008; Brockington et al., 2009). Flower colour, and size varies with species. Under favourable conditions, the plants grows so fast and in about six weeks' time they can be flowering. Flowers are normally found in a group at the end of the stem.

The origin of most of the species is still uncertain, Matthews et al. (1993) commented that, the origin of *Portulaca* species is uncertain as new data and interpretations make the theories of their origins questionable. However, uncollaborated literature points South America as the origin of most species as most of the demes there matches ancient descriptions and records. The species consists of two main clades based on phylogenetic analysis. The first group is the Opposite Leaf clade, which is further divided into African-Asian clade, and Australian clade. The second group is the Alternate Leaf clade which is divided into Oleracea clade, Umbraticola clade, and Pilosa clade (Ocampo and Columbus, 2012).

1.2 *Portulaca oleracea* L.

Most available literature is about *Portulaca oleracea* L. which is described as an invasive aggressive weed, in most agricultural settlements. Coquillant (1951) mentioned *P. oleracea* as the eighth most frequent weed (unwanted plant). It is currently distributed in all continents, and have some slight modifications depending on places. Its rapid spreading is a cause of concern to many farmers as it will cause competition to the main crop there by reducing yield. Ni HanWen et al. (2000) demonstrated the devastating effect of this species on the yield of maize (20 - 40% loss expected). Apart from them providing direct competition to the plants, they act as sources of some diseases as well as a host for insects. Salawu and Afolabi, 1994 reported *P. oleracea* as a host of *Meloidogyne incognita* in sugarcane plantations in Nigeria. Stevens et al. (1994) demonstrated that *P. oleracea* was a host of beet yellow virus.

Despite some of the negative uses, the plants in the genus *Portulaca* can also be used as cover crops to prevent erosion. *P. oleracea* is also used as a vegetable: purslane is one of the richest green plant sources of omega -3 fatty acids (Uddin et al., 2014). Methanolic extract of *P. oleracea* have been reported to have anti-oxidant activity (Sanja et al., 2009), and it is also considered to have antiphlogistic, bactericide, antidiabetic, anaphrodisiac, and diuretic effects, as well as being a refreshing agent (Boulous, 1983). Surface and emulsification properties of extracts from *Portulaca* family makes it much more suitable for hydrocolloids (Garti et al., 1999).

1.3*Portulaca umbraticola* Kunth

Two species namely *Portulaca grandiflora* Hook. and *Portulaca umbraticola* Kunth. have paramount ornamental value. Most literature involving ornamental *Portulaca* is about *P. grandiflora,* however, *P. umbraticola* is taking the floriculture industry by storm. *P. umbraticola* is an annual summer bedding plant with morphological characteristics that resemble those of common purslane (*P. oleracea* L.). It has recently become an important summer bedding plant in Japan due to its adaptability to the very weather conditions, a typical of the Japanese summer. Although the flower shape of *P. umbraticola* resembles that of *P. grandiflora,* its leaf morphology highly resembles that of common purslane (*P. oleracea*). As a result, *P. umbraticola* cultivars were named "HanaSuberihiyu" in Japanese, which means ornamental *P. oleracea* or were simply called "Portulaca", which is its genus name. *P. umbraticola* cultivars are frequently mislabeled as *P. oleracea* or *Portulaca grandiflora* or as hybrids of these species. A strain called Wildfire has been offered as both *P. grandiflor*a and *P. oleracea,* but it is actually a strain of *P. umbraticola* cultivars (Matthews et al., 1992).

Recently, Ocampo and Columbus (2012) illustrated that *P. umbraticola,* formerly referred to as *Portulaca* hybrid or *P. oleracea* subsp. *sativa,* belongs to the Umbraticola clade within the genus *Portulaca* and is indeed *P. umbraticola.* In *Portulaca*, well defined qualitative traits are few, the presence of a wing on the capsule is one such character which can be used to identify *P. umbraticola* species (Matthews et al., 1992). The genus *Portulaca* have species that exists in a myriad of local populations exhibiting morphological variations which are confined to specific demes. As a result consistent and persistent characters must be used for taxonomic classification, such characteristics include the wing pod as well as seed surface structure. These two characteristics are always distinct between *P. umbraticola* and *P.*

oleracea, regardless of the deme. The Wildfire Mixed was introduced by a Pan American company around 1983, though Mathews (1992) retained the name *P. umbraticola*, confusion of its classification was still an issue. As early as 1991, reports which augments that *P. umbraticola* was distinct from *P. oleracea* also emerged in Japan with Shibata (1991), reporting chromosome numbers of some HanaSuberihiyu cultivars instead of using the term Suberihiyu, thus at that point *P. oleracea* was already being clearly distinguished from *P. umbraticola* in Japan although the documentations does not mention its scientific name. Moreover, it seems the presence of larger flowers was the only characteristic used to distinct Suberihiyu (*P. oleracea)* from Hanasuberihiyu (*P. umbraticola*).

Complexity of classification and unavailability of literature for *P. umbraticola* prompted the need for more studies on the diversity of *P. umbraticola* cultivars. With the current trends of increase in global temperatures, mitigation and adaptation measures are crucial. The C_4 photosynthetic pathway of *Portulaca* plants allows them to thrive under high light intensity and strong heat with limited water supply (Kochy and Kennedy, 1982). Moreover, the genus consists of $C_4 - C_3$ intermediates and the species have both NADP-ME and NAD-ME C_4 pathway (Voznesenskaya et al., 2010) making them potential candidates for heat stress tolerance model plants.

Currently most of the cultivated *Portulaca (P. umbraticola* Kunth) is grown by means of cuttings as most of the cultivars are sterile. However, seeds seems to be also available for some cultivars. The plant has a wide tolerance of temperature, photoperiods, and light intensity though low temperatures are unfavourable. It can withstand temperatures of up to 40 ^{o}C. Light is extremely crucial for germination. There is no precise flowering photoperiod for *Portulaca* plants; however, overall plant growth and capsule production increases with day

length. For ornamental purposes the plants are normally grown as summer bedding plants, hanging in baskets as well as on rocky areas. Sometimes it can be used as a cover plant as it can easily spread over the surface due to its aggressiveness.

1.4 Introduction of *Portulaca umbraticola* cultivars to Japan and the development of their breeding

The first documentation concerning *Portulaca umbraticola* in Japan dated back to 1984 when a double flowered cultivar having yellow-violet chimeric flowers was reported as *P. oleracea* var. *granatus* (Shibata, 1984). Thereafter, *P. umbraticola* cultivar 'Wildfire' was reported as *P. oleracea* (Shibata, 1989). The report gives a brief introduction and description of the cultivar 'Wildfire': it states that the cultivar was released for sale as ornamental *Portulaca* in the United States around 1982, the cultivar is described as having single flower types (2.5 cm flower diameter), yellow, pale pink, and salmon pink in flower color and the stems can spread up to 70 cm. It was also stated that the seeds were sterile. Besides the single type flowers of 'Wildfire' cultivar, some double flower types were also said to be available. *P. umbraticola* cultivars are frequently mislabeled as *P. oleracea* or *Portulaca grandiflora* or as hybrids of these species. A strain called Wildfire has been offered as both *P. grandiflor*a and *P. oleracea,* but it is actually a strain of *P. umbraticola* cultivars (Matthews et al., 1992). In all the cases above, the description and characteristic features were a match but different names were used, suggesting the complexity of naming *P. umbraticola* cultivars in the past. The only slight variation in the descriptions was the diameter of the flowers.

Matthews (1992), a professor at the University of North Carolina in US made some tremendous efforts in trying to clear the naming confusion retaining the name *P. umbraticola* for the cultivar 'Wildfire Mixed'. In his work 'Wildfire Mixed' is said to have been

introduced by Pan American Seed Company in 1982-83, having unstable flower color. Efforts were made to fix flower color by breeding, but it failed due to remarkable inbreeding. It is important to note that the description by Shibata (1989) was a near perfect match to the 'Wildfire Mixed' described in Matthews work. Matthews also made it easier on how to distinguish *P. umbraticola* from *P. grandiflora* and *P. oleracea* as well as other species. *P. umbraticola* cultivars have a wing pod on the capsule and consist of stellate seed surface cells. These features are consistent and confined to this species, recent research also supports Matthews's arguments with Ocampo and Columbus (2012), being able to distinguish *P. oleracea* from *P. umbraticola* through phylogenetic studies.

Currently, most of the popular *P. umbraticola* cultivars in Japan, were brought by Mr. Kunihiko Hido, who purchased those cultivars from a German farm in 1983, and introduced them to Japan. The cultivars are propagated by means of rooted cuttings due to the sterile nature of their seeds, it is important to note that recently most of these cultivars have large flower size in comparison to conventional cultivars. Mr. Hido suggests that increases in flower size and variation of colors are as a result of mutations as well as crossing (hybridization) of different dimes of *P. umbraticola*. He also stated that Mr. Hiromi Matsukizono, a breeder in Kagoshima Prefecture, contributed greatly to the modification of the current cultivars in Japan through crossing. He released many new varieties which have larger flower diameter, bicolored, double flowers, and have variegated leaves named 'Yubi' series. In 2012 Sakata Seed Co. Ltd. also released new varieties 'Sanchuraka' series, which have better flower longevity than conventional cultivars. Kanno (2012) reported that several newly bred cultivars have flowers that are open till late evening unlike the conventional types. As a result of different great breeding efforts, a lot of new cultivars which have larger flower size, better flower longevity, and plenty of colours have been released. However, most of

these characteristic are yet to be precisely examined.

1.5 Research Purpose

The main purpose of this research was to establish the basis for future improvement of ornamental *Portulaca*. A lot of cultivars have been recently released (most of them obtained through crossing of different populations). Such hybridisation is known to promote the occurrence of polyploidy, which is a major force in promoting variation. In contrast to conventional cultivars, these new cultivars have more desirable characteristics. So I designed experiments to determine the ploidy levels of the newly released cultivars. I also wanted to check if there was any correlation between the determined ploidy levels and flowering characteristics such as flower diameter and flower longevity. Flowers of polyploidy plants have thicker petals which results in longer lasting flowers (Ranney, 2006). The degree of ploidy level has been known to correlate with organ size; in *Lolium* species tetraploids had larger leaf size, mesophyll, and epidermal cells compared to diploids (Sugiyama, 2005). Most species of the genus *Portulaca* are known to have small flower diameters, as such ways to improve flower size will be of great value and knowledge of ploidy levels and their correlation to organ size might be useful. Secondly, I wanted to investigate the differences between conventional cultivars and currently released new cultivars with an aim of improving flowering characteristics and understanding various physiological processes related to flowering. Thus I investigated the mechanisms controlling the timing of flower opening, I also checked for factors affecting flower senescence and probable ways to improve flower longevity.

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*. As a result, I wanted to assess the effect of different photoperiods on flower opening time in *P. umbraticola*. I also wanted to investigate the involvement of the plant circadian clock in *P. umbraticola* flower opening, as well as the response of the plant to different light qualities in relation to flower opening rhythms.

More importantly I wanted to develop comprehensive gene expression information of *P. umbraticola* by high-throughput RNA-sequencing which will facilitate molecular studies, which are vital for understanding the various physiological process/responses observed through physiological experiments. Expressed sequence tags (ESTs) are used for gene mining, and I was particularly interested in finding transcripts associated with the ethylene biosynthesis, ethylene perception and signalling pathway as well as the plant circadian clock related transcripts. Having already found out that senescence seemed to be ethylene depended, I wanted to characterise the expression of the ethylene related transcripts and find their correlation with senescence. Several studies have shown that silencing some of these ethylene related transcripts significantly improves longevity of flowers which show ethylene dependant senescence. Moreover, through physiological experiments, I found that flower opening is under the control of a circadian clock however, most of our conclusions were based on the assumption that the clock mechanism of *P. umbraticola* did not drastically differ from that of *Arabidopsis* hence it was necessary to verify the assumptions.

Fig. 1.1 *Portulaca umbraticola* and *Portulaca oleracea* plants.

Left: *P. umbraticola* cultivar ‘Single Red’, Right: *P. oleracea*

CHAPTER 2

[redacted]

本章の内容は、学術雑誌論文として出版する計画があるため公表できない。

５年以内に出版予定

CHAPTER 3

Difference in Flower Longevity and Endogenous Ethylene Production of *Portulaca umbraticola* Cultivars

3.1 Introduction

In the genus *Portulaca*, two species are mainly used for ornamental purposes, that is, *Portulaca grandiflora* Hook. and *Portulaca umbraticola* Kunth. The flowers of *Portulaca* are ephemeral and generally open early in the morning and wilt in the afternoon; the flowers do not fully open on cloudy or relatively cool days (Ichimura and Suto, 1998a). The ephemeral nature of *Portulaca* flowers has greatly affected their use as an ornamental plants. Recently, there have been an increase in the sales of ornamental *Portulaca* spp., with *Portulaca* being ranked as the 9th most grown summer bedding plant in Japan as of 2009 (Japan Flower Promotion Centre Foundation; http://www.jfpc.or.jp/bunseki/ 2009.html, September 1, 2010).

Although there has been an increase in the sales of floricultural *Portulaca*, there is little information about the flowering characteristics of these plants. *Portulaca* umbraticola, which was named as *Portulaca* hybrid in the work of Ichimura and Suto (1998b), grows well under very hot and dry conditions typical of a Japanese summer. The average life of *P. umbraticola* is greatly affected by light and temperature (Ichimura and Suto, 1998b), with high temperature and light facilitating rapid flower opening but also early closing. In *P. umbraticola*, pollination, filament wounding and pistil removal significantly accelerated senescence, with filament wounding being much more effective. Filament wounding increased ethylene production (Ichimura and Suto, 1998a). Iwanami and Hoshino (1963) also reported that filament wounding in *P. grandiflora* accelerated senescence.

Recently, a lot of new cultivars have been bred. Sakata Seed Co. ltd released a new series of *P. umbraticola* cultivars in 2012 known as the 'Sanchuraka series' The Sachuraka series cultivars appeared among cross seedlings independently, and were not obtained by spontaneous mutation (Kanno, 2012). There is little to no information about the flowering characteristics of these newly released cultivars. Thus in this study, we try to identify the main differences between a conventional cultivar, 'Single Red' (SR), and a newly released cultivar, 'Sanchuraka Cherry Red' (SCR), in relation to ethylene sensitivity, senescence and flower longevity.

SCR belongs to the Sanchuraka series, while SR is a conventional cultivar similar to *P. umbraticola* 'ANR1' used by Ichimura and Suto (1998a, b), the only difference being the name used. General observations have shown that 'Sanchuraka' series cultivars last longer than conventional cultivars; however, there is no specific information on their longevity as well as the mechanism used for their selection. This research should provide the basic information necessary for future improvement of ornamental *Portulaca* as well as providing an insight into the possible mechanism used for the selection of cultivars with better flower longevity. The main objectives were:

1. To determine the difference in flower longevity of *P. umbraticola* cultivars
2. To determine factors affecting flower longevity in *P. umbraticola* cultivars
3. To understand the mechanism of senescence of *P. umbraticola* cultivars

1.2 Materials and Methods

3.2.1 Plant materials and growth conditions

Portulaca umbraticola cultivars ‘Single Red’ (SR) and ‘Sanchuraka Cherry Red’ (SCR) were mainly used in this experiment. The plants were raised at similar conditions to the one describe in chapter 2.2.2

3.2.2 Flowering characteristics

3.2.2.1 Determination of flower longevity

The flower buds of SR and SCR were harvested a day before anthesis and then placed into test tubes containing distilled water. The buds were stored in a refrigerator overnight at 10°C. The following morning at 8 a.m., the flowers were transferred into the phytotron and the time taken from opening to closing was recorded using a digital camera at 5-min intervals. A total of five flower buds were used for each experiment.

Apart from that, the effects of temperature on flower opening were also evaluated. The above procedure was followed until the following morning. In the morning, the buds were then subjected to different temperatures (22.5°C–40°C) in a growth chamber (LH-240NPFLED; Nippon Medical and Chemical Instruments Co. Ltd., Osaka, Japan) with light intensity of 100–105 μmol· m^{-2}·s^{-1}. The time taken to full opening was recorded. A total of five flower buds were used for each experiment.

3.2.2.2 Effects of pollination, pistil removal and filament wounding on flower longevity

The above procedure was followed and most flowers opened within three hours. The flowers that did not open within this time period were discarded. The flowers were then subjected to self/cross-pollination; for pistil removal, the pistil was carefully pulled out by hand. The filaments were wounded by carefully cutting them to about 1/3 from their base using a pair of tweezers, following the method used by Ichimura and Suto (1998a). Untreated plants were used as a control. A total of five flower buds were used for each experiment.

3.2.3 Ethylene treatments

3.2.3.1 Determination of ethylene produced from flower opening to closing by the two cultivars.

The flower buds were harvested a day before anthesis and then stored in a plant growth chamber (FLI-2000H; EYELA, Tokyo, Japan) of 28/25°C day and night temperatures, respectively, with cool fluorescent light (315–325 $\mu mol \cdot m^{-2} \cdot s^{-1}$) and 70% RH. The lights were turned on at 7 a.m. and turned off at 7 p.m. Ethylene production was measured at 2-h intervals from opening to closing. Two flowers per cultivar were sealed in a 25-mL flask and incubated for 30 min at the above temperature. Head space gas samples (2 mL) were taken and injected into a gas chromatograph (GC-7A; Shimadzu, Kyoto, Japan) equipped with an alumina column and a flame ionization detector. This was replicated three times giving a total of six flowers per measurement.

3.2.3.2 Exogenous ethylene treatment

Flower buds were held under the above conditions and fully open flowers were selected at 9 a.m. The flowers were placed in sealed incubation chambers with a small circulating fan. Designated ethylene concentrations (0.5, 1, and 2 $\mu L \cdot L^{-1}$) were introduced by a syringe through an inlet rubber-sealed pipe. The flower were incubated for 1 h. Untreated plants were used as a control. A digital camera was used to record the time taken to wilting. A total of eight flowers were used for each experiment.

3.2.3.3 Ethylene action and biosynthesis inhibitor treatments (AVG and 1-MCP)

Flower buds were held under the above conditions until they were fully open. For aminoethoxyvinylglycine (AVG) treatments, 5 $\mu L \cdot L^{-1}$ AVG (SigmaAldrich, St. Louis, MO, USA) was spread on the filaments of the flowers using a syringe (5 mL/flower). For 1-methylcyclopropene (1-MCP), flowers with their cut ends immersed in distilled water were placed in an acrylic chamber (53 L), and then Ethyl Bloc TM (Rohm and Haas Japan, Tokyo, Japan) was added to the distilled water to evolve 1-MCP at a concentration of 2 $\mu L \cdot L^{-1}$ and held at 28°C for 1 h. Untreated plants were used as a control. Time taken to wilting was recorded using a digital camera. A total of five flower buds were used for each experiment.

Statistical analysis

The data were subjected to analysis of variance (ANOVA) using a statistical package, R-Console (Rx64 3.0.2) (https://www.r-project.org/).

Fig. 3.1 *Portulaca umbraticola* cultivars used in most of the studies.

Left: Cultivar ‘Sanchuraka Cherry Red’ (SCR) (it have erect stems)

Right: Cultivar ‘Single Red’ (SR) (it have crippling stems)

3.3 Results

3.3.1 Difference in flower longevity

The flowers of SR took 1.2 h to open fully from the bud stage, while those of SCR took 2.5 h. However, SR took 10.3 h from the bud stage to closing, while SCR took 15.3 h **(Table 3.1).** SR and SCR significantly differed in their flower longevity, having averages of 9.1 and 12.8 h, respectively.

At 22.5 and 25°C, the flowers of both cultivars did not fully open. After 6 h at 25°C, SR was at a more advanced opening stage than SCR, but the flowers never reached the fully open stage (data not shown). A temperature of 27.5°C was the minimum threshold for opening as both cultivars reached the fully open stage; SR took 3.3 h while SCR took 4.4 h. The difference in opening time between the two cultivars decreased with an increase in temperature. At 30°C, SR took 3.2 h to open fully while SCR took 4 h. At 32.5°C, SR took 3.0 h while SCR took 3.5 h. At 35°C, 37.5°C, and 40°C, the differences in time taken to full opening were not significant **(Table 3.2),** with SR taking 2.6, 2.9, and 2.5 h, respectively, while SCR took 2.7, 3.2, and 2.8 h. At 40°C, the petals appeared slightly droopy, indicating that the temperature was too high for the flowers.

3.3.2. Effects of pollination, pistil removal, and filament wounding on flower longevity

Pollination and pistil removal significantly accelerated senescence in both cultivars. However, filament wounding was much more effective in accelerating the rate of senescence. In SR, the control recorded 9 h, while the cases with pollination and pistil removal recorded 6 and 6.9 h, respectively **(Table 3.3).** Filament wounding had the greatest effect, recording 3.9 h. In SCR, the control recorded 11.9 h, while the cases with pollination and pistil removal recorded 7.8 and 8 h, respectively. Just like in SR, filament wounding also had the greatest effect in SCR, reducing the flower longevity to 5.4 h **(Table 3.3).**

3.3.3. Ethylene produced from flower opening to closing

The flowers reached the fully open stage after 2 h, which is when ethylene measurement started. At 2 h, SR produced 1.97 $nL \cdot g^{-1}$ $FW \cdot h^{-1}$ ethylene, while SCR produced 1.95 $nL \cdot g^{-1}$ $FW \cdot h^{-1}$. At 4, 6, and 8 h, SR produced slightly less ethylene than SCR, recording 2.40, 2.40, and 3.80 $nL \cdot g^{-1}$ $FW \cdot h^{-1}$, while at those times, SCR produced 3.36, 3.50, and 3.96 $nL \cdot g^{-1}$ $FW \cdot h^{-1}$, respectively **(Fig. 3.2)**. Thereafter, SR had a sharp and significant rise at 10 h, when it reached its peak of 13.65, while SCR recorded 3.67 $nL \cdot g^{-1}$ $FW \cdot h^{-1}$. SCR reached its peak 2 h later at 12 h, which was 5.36; at this point, SR was fully closed and produced its last recorded amount of ethylene, which was 10.14 $nL \cdot g^{-1}$ $FW \cdot h^{-1}$ and was also significantly higher than the SCR peak. SCR was almost closed at 14 h, when it produced its last recorded amount of ethylene, which was 4.42 $nL \cdot g^{-1}$ $FW \cdot h^{-1}$ **(Fig. 3.2).** Both SR and SCR produced detectable amounts of ethylene from opening to closure. The peak levels of ethylene production by the two cultivars were significantly different, with SR producing

more ethylene than SCR. The peak ethylene production in SR occurred at 10 h, which was 2 h earlier than the peak of SCR, occurring at 12 h **(Fig. 3.2).**

3.3.4. Exogenous ethylene treatment

In SR, exogenous ethylene treatment accelerated senescence. A level of 0.5 $\mu L \cdot L^{-1}$ resulted in flowers wilting at 6.5 h, while cases with 1 and 2 $\mu L \cdot L^{-1}$ recorded 6.4 h **(Table 3.4).** Exogenous ethylene was much more effective at accelerating senescence than the control, for which the time of wilting was 9.3 h. In SCR, the trends were almost the same as for SR. SCR control recorded 11.9 h, while 0.5 and 1 $\mu L \cdot L^{-1}$ had the same flower longevity of 8.5 h. The case of 2 $\mu L \cdot L^{-1}$ had slightly shorter flower longevity, recording 8.4 h **(Table 3.4).** Exogenous ethylene treatments significantly accelerated senescence in both cultivars. There was no significant difference among the three concentrations used (0.5, 1, and 2 $\mu L \cdot L^{-1}$).

3.3.5. Ethylene action and biosynthesis inhibitor treatments (AVG and 1-MCP)

1-MCP, an ethylene action inhibitor, significantly improved flower longevity in both cultivars. It seems its effect was much greater in SR, which averaged 13.8 h compared with 9 h for the control. In SCR, it recorded 13.0 h, compared with 10.4 h for the control. AVG, an ethylene biosynthesis inhibitor, had a much more significant effect, improving the longevity of both cultivars. In SR, AVG-treated flowers had average flower longevity of 22.1 h, while in SCR, they had 24.6 h **(Table 3.5).**

Table 3.1. Difference of flower longevity of *P. umbraticola* cultivars.

Cultivar	Time taken to open (h)	Time taken to close (h)	Flower longevity (h)
SR	1.2 ± 0.3 **	10.3 ± 0.1 **	9.1 ± 0.2 **
SCR	2.5 ± 0.1	15.3 ± 0.2	12.8 ± 0.3

** indicates significant difference in *t*-test ($P < 0.01$, n = 5). The times taken to open and to close are also shown; this time is from the bud stage.

Values are means ± SE.

Table 3.2. Effects of temperature on flower opening of *P. umbraticola* cultivars.

Cultivar	Time to open at different temperatures (h)					
	27.5℃	30℃	32.5℃	35℃	37.5℃	40℃
SR	3.3 ± 0.1	3.2 ± 0.2	3.0 ± 0.1	2.6 ± 0.6	2.9 ± 0.3	2.5 ± 0.2
SCR	4.4 ± 0.3	4.0 ± 0.2	3.5 ± 0.1	2.7 ± 0.2	3.2 ± 0.7	2.8 ± 0.4
	*	*	*	n.s.	n.s.	n.s.

* indicates significant difference in *t*-test ($P < 0.05$, n = 5); n.s. indicates no significant difference. Values are means ± SE.

Table 3.3. The effects of pollination, pistil removal and filament wounding on flower longevity of *P. umbraticola* cultivars.

Cultivar	Treatment	Time taken to close (h)	
SR	Control	9.0 ± 0.4	a
	Pollination	6.0 ± 0.3	c
	Pistil removal	6.9 ± 0.3	b
	Filament wounding	3.9 ± 0.2	d
SCR	Control	11.9 ± 0.5	a
	Pollination	7.8 ± 0.3	b
	Pistil removal	8.0 ± 0.3	b
	Filament wounding	5.4 ± 0.2	c

Different letters indicates significant differences in Tukey's multiple range test ($P < 0.01$, n = 5). Values are means ± SE.

Table 3.4. The effects of exogenous ethylene treatment on flower longevity of *P. umbraticola* cultivars.

Cultivar	Ethylene treatment (µL/L)	Time taken to close (h)	
SR	Control	9.3 ± 0.2	a
	0.5	6.5 ± 0.6	b
	1	6.4 ± 0.6	b
	2	6.4 ± 0.4	b
SCR	Control	11.9 ± 0.6	a
	0.5	8.5 ± 0.3	b
	1	8.5 ± 0.3	b
	2	8.4 ± 0.3	b

Different letters indicates significant differences in Tukey's multiple range test ($P < 0.01$, n = 8). Values are means ± SE.

Table 3.5. The effects of ethylene inhibitor treatments on flower longevity of *P. umbraticola* cultivars.

Cultivar	Ethylene inhibitor	Time taken to close (h)	
SR	Control	9.0 ± 0.3	c
	AVG	22.1 ± 2.0	a
	1-MCP	13.8 ± 0.4	b
SCR	Control	10.4 ± 0.3	c
	AVG	24.6 ± 3.0	a
	1-MCP	13.0 ± 0.7	b

Different letters indicate significant differences in Tukey`s multiple range test ($P < 0.01$, n = 8). AVG: aminoethoxyvinylglycine, 1-MCP: 1-methylcyclopropene. Values are means ± SE.

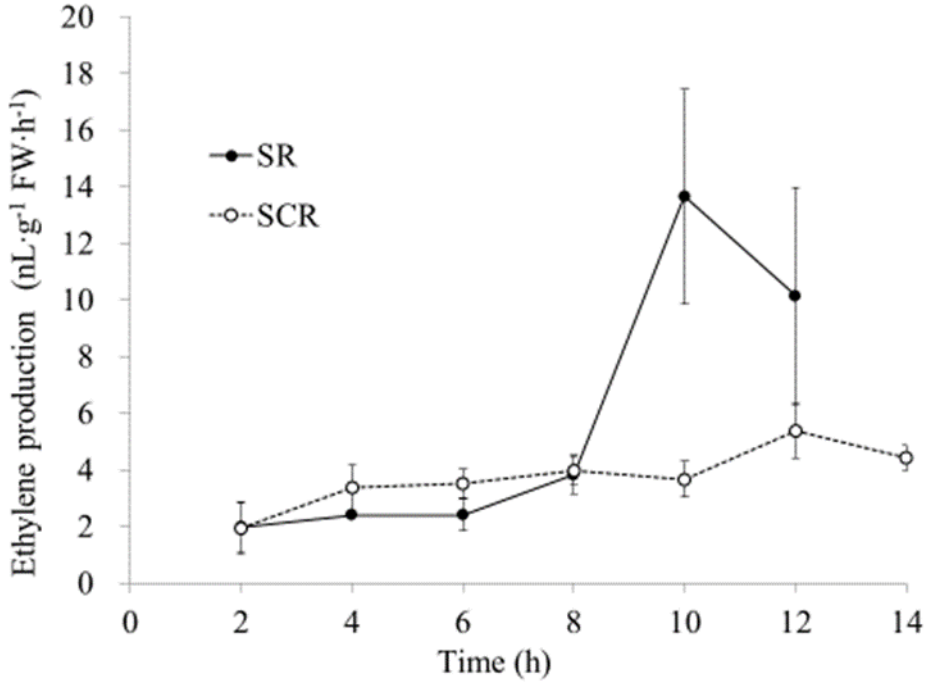

Fig. 3.2 Endogenous ethylene production from flower opening to flower closure of *P. umbraticola* cultivars SR: 'Single Red' and SCR 'Sanchuraka Cherry Red'.

The flowers of SR were fully closed at 12 h, while those of SCR were almost closed at 14 h. Sampling was done at 2 h intervals, starting 2 h after the light on signal (light on was at 0 h) Data are presented as the mean ± SE (n = 3).

3.4 Discussion

SCR and SR had different flower longevities of 12.8 and 9.1 h, respectively **(Table 3.1).** In flowering plants, flower longevity is species-specific and is closely linked to reproductive strategy (Shibuya et al., 2014). The newly released 'Sanchuraka' cultivars also showed some slight variation in flower longevity among themselves (data not shown). The difference in flower longevity between SR and SCR might have been due to the difference in the amount of endogenous ethylene produced. In carnation, the long-lasting cultivars produced less ethylene than short-lived conventional cultivars (Nukui et al., 2004; Onozaki et al., 2006). In *P. umbraticola*, SR produced more ethylene (with peak ethylene production of 13.65 $nL \cdot g^{-1}$ $FW \cdot h^{-1}$) than SCR (5.36 $nL \cdot g^{-1}$ $FW \cdot h^{-1}$) **(Fig. 3.2).** Moreover the peak of endogenous ethylene production was approximately 2 h earlier in SR than in SCR. The flowers of SCR opened and closed later than those of SR. SCR took 15.3 h from opening to closure, while SR took 10.3 h **(Table 3.1).** SCR requires a high temperature to open compared with SR **(Table 3.2)**; at 25°C, although SR did not fully open, it was at a more advanced opening stage than SCR, probably showing that, at a lower temperature than for SCR, SR can accumulate enough heat to open.

Pollination and pistil removal significantly accelerated senescence of both SCR and SR; however, filament wounding was much more effective in accelerating senescence. In *P. umbraticola*, filament wounding accelerated senescence by increasing ethylene production (Ichimura and Suto, 1998b). Pollination has been reported to accelerate senescence in many flowers such as orchids (Porat et al., 1994) and *P. grandiflora* (Iwanami and Hoshino, 1963). In Petunia, pollination and wounding of pistils stimulated senescence (Whitehead et al., 1984). The effects of pollination, pistil removal and filament wounding may be attributable to

an increase in ethylene production since there will be small chances of wounding, which in turn can cause ethylene production. Our results agree with the findings of Ichimura and Suto (1998b) using the conventional cultivar ANR1, although this previous paper refers to it as *Portulaca* hybrid.

Exogenous ethylene treatments at 0.5, 1, and 2 $\mu L \cdot L^{-1}$ significantly accelerated the rate of senescence in both cultivars **(Table 3.4).** There was no significant difference between the three ethylene concentrations used. Since exogenous ethylene accelerated senescence in both cultivars, this implies that the long vase life of SCR is probably associated with lower endogenous ethylene production rather than lower ethylene sensitivity. However, the ethylene treatments might have been above the threshold, and it was practically challenging to designate concentrations of below 0.5 $\mu L \cdot L^{-1}$, which would probably reveal if they are any differences in sensitivity. In carnations, flowers of lines selected for a long vase life produced very low levels of ethylene during senescence, whereas the ethylene sensitivity of the selected lines was generally high (Onozaki et al., 2006). Although *P. umbraticola* is ephemeral in nature, our results can be compared to those of carnations. The use of AVG and 1-MCP significantly improved flower longevity in both cultivars. Since exogenous ethylene accelerated senescence and AVG and 1-MCP delayed senescence, this confirmed that the senescence of *P. umbraticola* cultivars SR and SCR is ethylene-dependent. In *Hibiscus rosa-sinensis*, treatment with ACC and 1-MCP confirmed that flower senescence in *Hibiscus* is ethylene-dependent (Trivellini et al., 2011). In *Portulaca* hybrid, treatment with ethylene for one hour significantly accelerated flower senescence, and the senescence of both intact and filament-wounded flowers was markedly delayed by exposure to norbornadiene (NBD), an inhibitor of ethylene action (Ichimura and Suto, 1998a). In *Ipomoea nil* 'Violet', exogenously applied ethylene accelerated petal senescence; however, AVG and 1-MCP did not delay

senescence (Yamada et al., 2006). On the basis of these observations, petal senescence of *I. nil* 'Violet' is considered to be regulated independently of endogenous ethylene (Shibuya, 2012; Yamada et al., 2006). Although the senescence of both *P. umbraticola* cultivars and *I. nil* 'Violet' is accelerated by exogenous ethylene, their senescence pathways tend to differ since they respond differently to ethylene action and ethylene biosynthesis inhibitors. The plant growth hormone ethylene is known to hasten the senescence of many cut flowers such as carnations (Mayak et al., 1976; Satoh, 2011) and *Portulaca* hybrid (Ichimura and Suto, 1998a). This was almost the same scenario as how exogenously applied ethylene accelerated senescence of both SR and SCR. Senescence of *P. umbraticola* cultivars is ethylene-dependent, so ethylene inhibitors prolonged vase life while exogenous ethylene accelerated senescence.

In conclusion, flower longevity of SCR might have been selected on the basis of endogenous ethylene production rather than ethylene sensitivity. Both SR and SCR were sensitive to exogenous ethylene, but SCR produced less ethylene than SR, thereby probably explaining the difference in their flower longevity. However, the mechanism through which SCR produces lower endogenous ethylene than SR is still not clear. Furthermore, analysis of ethylene-associated genes from flower opening to closure might be crucial for understanding the senescence process. To obtain a deep understanding of this there is a need for information specific to each cultivar, rather than generalizing the findings across the species. With the current trend of a global rise in temperature, *P. umbraticola* provides a realistic adaptation option in the ornamental industry, so basic information on this ornamental plant is promising for promoting the future of the industry

CHAPTER 4

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

4.1 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 PHYTOCROME 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. 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, I 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.

4.2 Materials and Methods

4.2.1 Plant Materials

P. umbraticola cultivar 'Single Red' (SR) was used as experimental material in all experiments. This cultivar is almost sterile, it was vegetatively propagated by means of cuttings. The plants were raised in a similar way to the description in previous chapters. Potted plants bearing many flower buds **(Fig. 4.1A)** 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 %.

4.2.2 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 **Fig. 4.1 B**. Stage 2 of flower opening was used as the reference point (flower opening stage for these experiments) since this stage was not significantly influenced by light or darkness **(Fig. 4.2).** In contrast, the progression of flower opening from stage 2 onwards was greatly influenced by the presence or absence of light **(Fig. 4.2).** Thus, flower opening in this paper refers to stage 2. The images were captured for four consecutive days and all the treatments 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. 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.

4.2.3.1 Experiment 1. Effect of light exposure on flower opening

P. umbraticola buds were harvested directly from the phytotron at stage 1 (see **Fig. 4.1B**) 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 **Fig. 4.1B**. For flower closure, the flowers were assigned stage numbers (7, 8, 9, 10, and 11), these stages were analogous to the opening stages (5, 4, 3, 2, and 1), respectively (see insert on **Fig. 4.2).** 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.

4.2.3.2 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.

4.2.3.3 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).

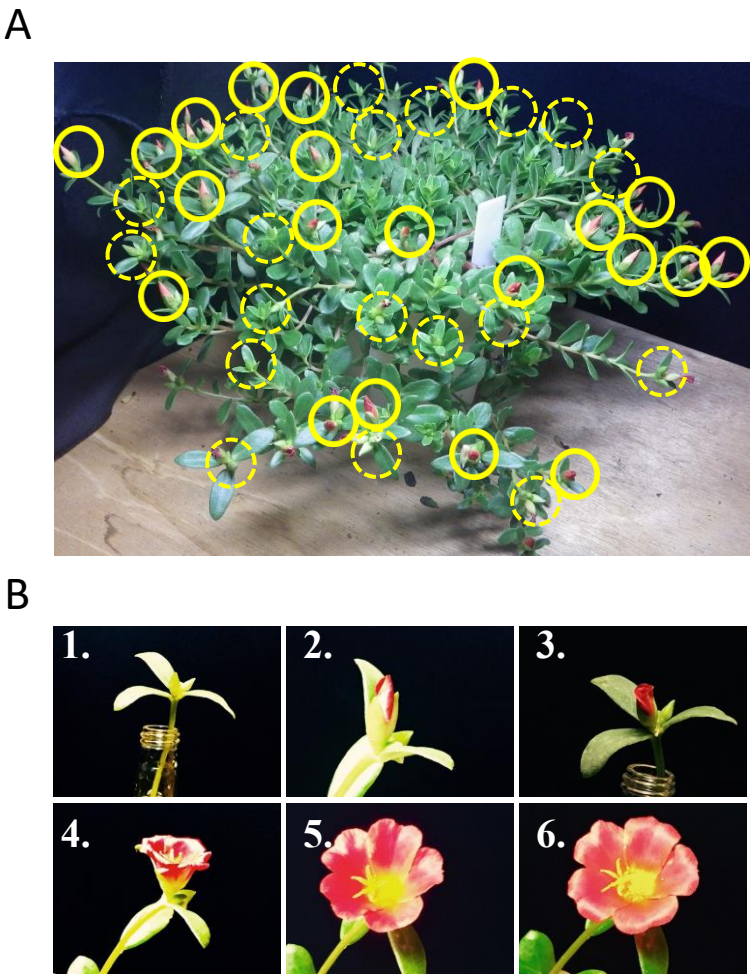

Fig. 4.1 *Portulaca umbraticola* plant used in this study.

(A) Image of a potted plant bearing many flower buds. Solid circles indicate opening buds and dotted circles indicate buds that will open from the second day onwards. **(B)** Stage number of flower opening in *P. umbraticola* cultivar 'SR'. The degree of opening was assessed by comparison with the stages shown. 1. Bud, a day before anthesis; 2. Petals emerge from the sepals and their length is almost the same as that of the sepals (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 separates at the top 5. Opening progresses and filaments can be clearly seen; 6. Full open flower.

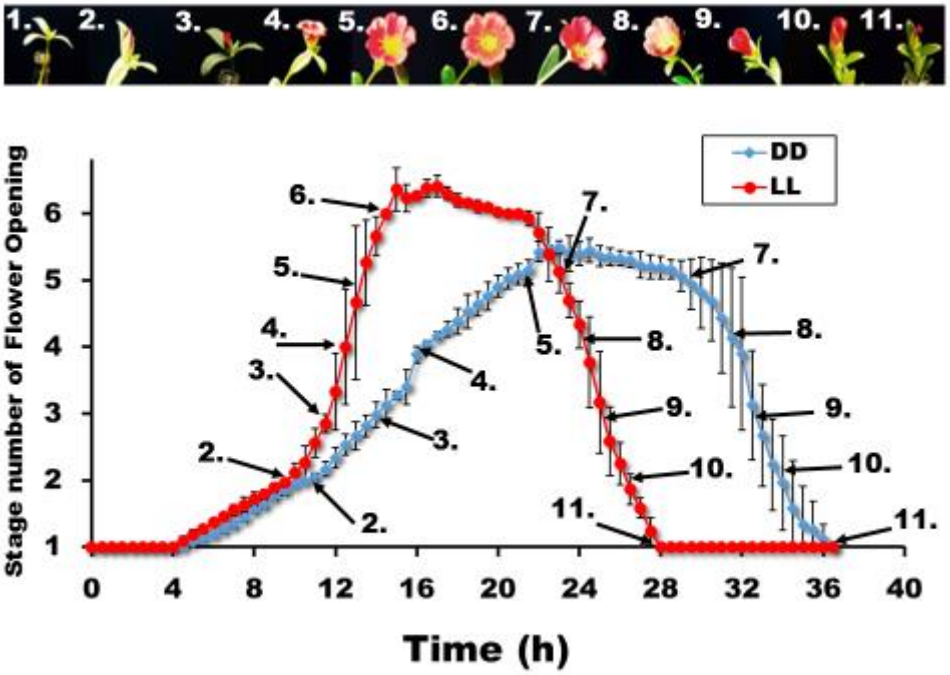

Fig.4.2 Progression of flower opening and closing under DD and LL**.**

Numbers (1-6) represent flower opening stages and they corresponds to the ones shown in **Fig. 1B**, (7-11) indicates stages of flower closure and they are analogous to opening stages (5-1), respectively. (See inserted picture on top of the graph). Data are means of 3 separate replications. Error bars represents standard deviation

4.3 Results

4.3.1 Differences in flower opening process under continuous light or darkness

Flower opening in *P. 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. 4.2),** 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. 4.2).** 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. 4.2 and 4.3).** The flowers in LL closed about 10 h earlier than those in DD **(Fig. 4.2).**

4.3.2 Effect 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. 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 *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. 4.3A).** 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. 4.3C** and **4.2**). 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. 4.3B** 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. 4.3B**). 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. 4.3D and 4.2).** 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. 4.4A**). 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. 4.4C**). The rhythm in RR was almost the same as that in LL (**Figs. 4.4A and 4.3B**). 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. 4.4D**). The flowers opened on almost the same CT on all three days.

4.3.3 Effect of different photoperiods on the time of flower opening

The potted plants with many flower buds were subjected 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. 4.5)**. 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. 4.5)**. 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. 4.5).**

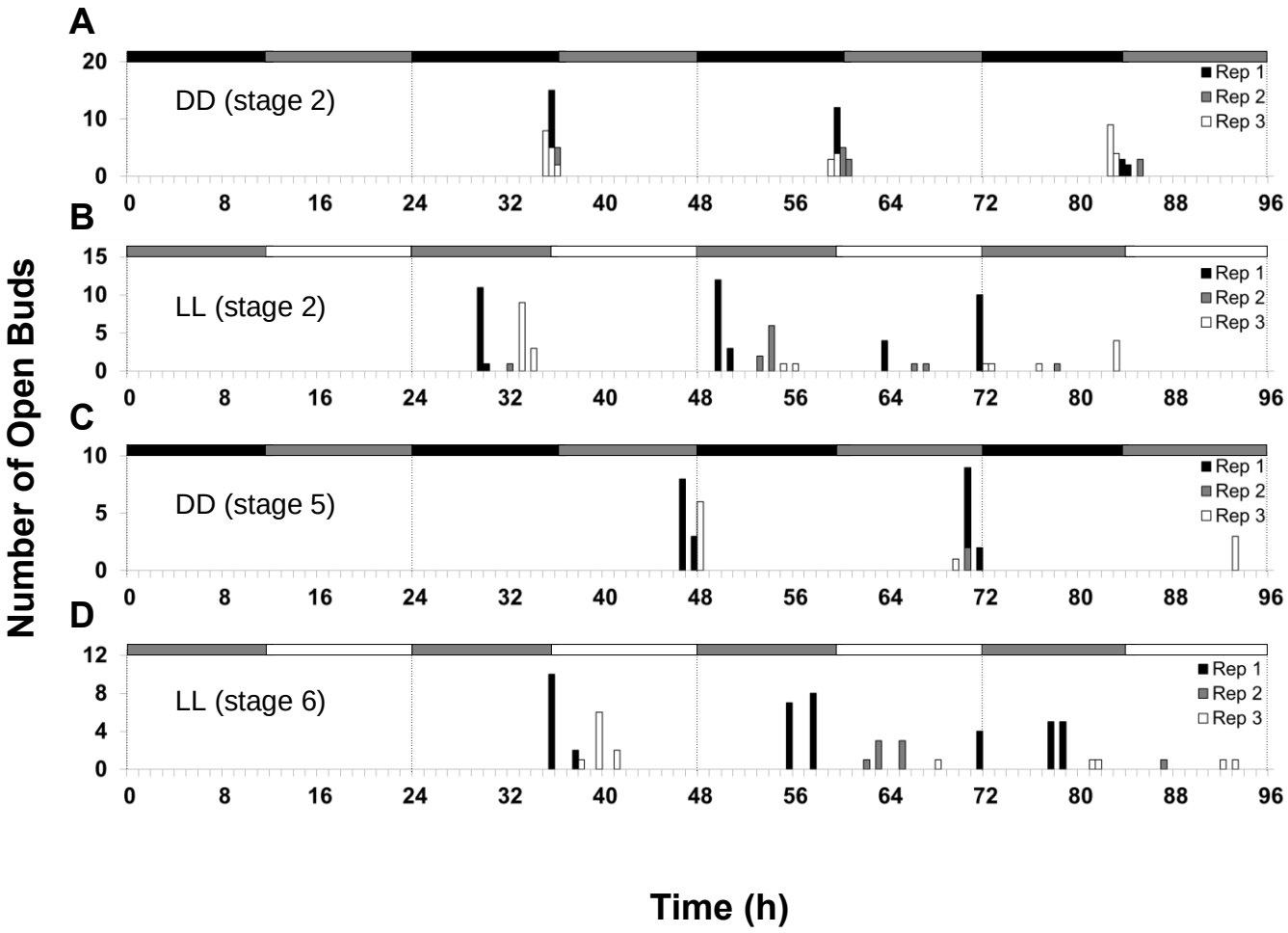

Fig. 4.3 Flower opening rhythm under constant conditions.

(A) rhythm in DD, **(B)** Rhythm in LL, **(C)** and **(D)** progression of flower opening in DD and LL, respectively. **(A)** and **(B)** are stage 2 of flower opening while **(C)** is stage 5 and **(D)** is stage 6. Black and grey bars in **(A)** and **(C)** represent subjective night and day, respectively. White and grey bars in **(B)** and **(D)** 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.

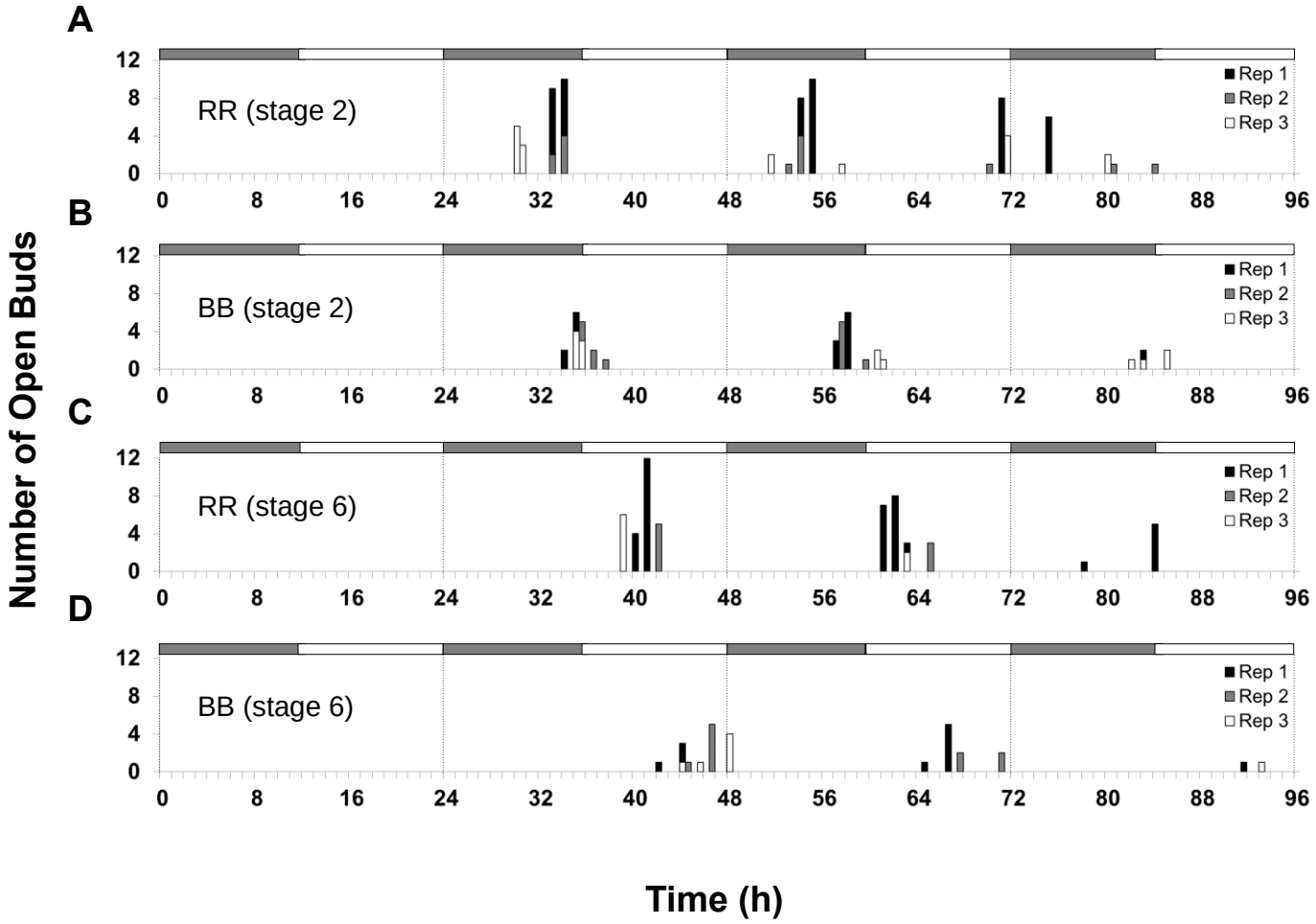

Fig. 4.4 Flower opening rhythm under constant conditions.

(A) rhythm in RR, **(B)** rhythm in BB, **(C)** and **(D)**progression of flower opening in RR and BB, respectively. **(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.

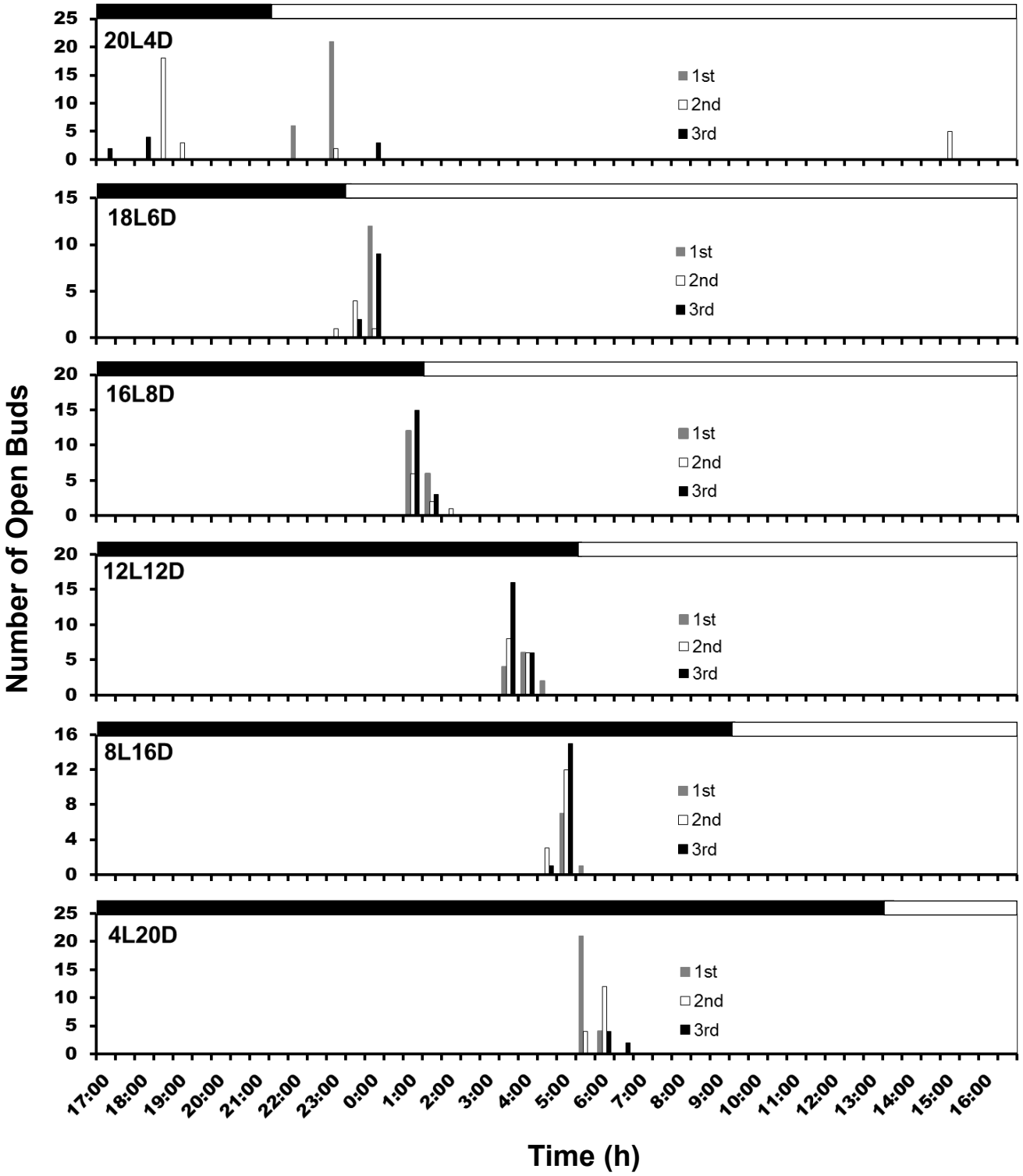

Fig. 4.5 Effect of different photoperiods on flower opening time.

1st, 2nd, and 3rd indicates the days on which the flower buds opened

4.4 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. 4.3A**), 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. 4.3B**). 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 regulate the rhythm of flower opening are activated in darkness. This was also suggested for the expression rhythm of floral inducer

genes in *Pharbitis* (*Ipomoea*) *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 lightintensity 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. 4.3A, B, and 4.4A**), 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. 4.3B** and **4.4A),** 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. 4.2, 4.3C, 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; Bieleski 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. 4.5 and 4.3B**). 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. 4.5**). 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. 4.5)** just like in LL (**Fig. 4.3B**). After this observation, I experimented with different photoperiods and I 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 (Bieleski 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. 4.5).** 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 I also subjected the plants to different cycles starting at different times and recognised that I 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, I can regulate flower opening to our desired time.

4.4.1 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. 4.6)**

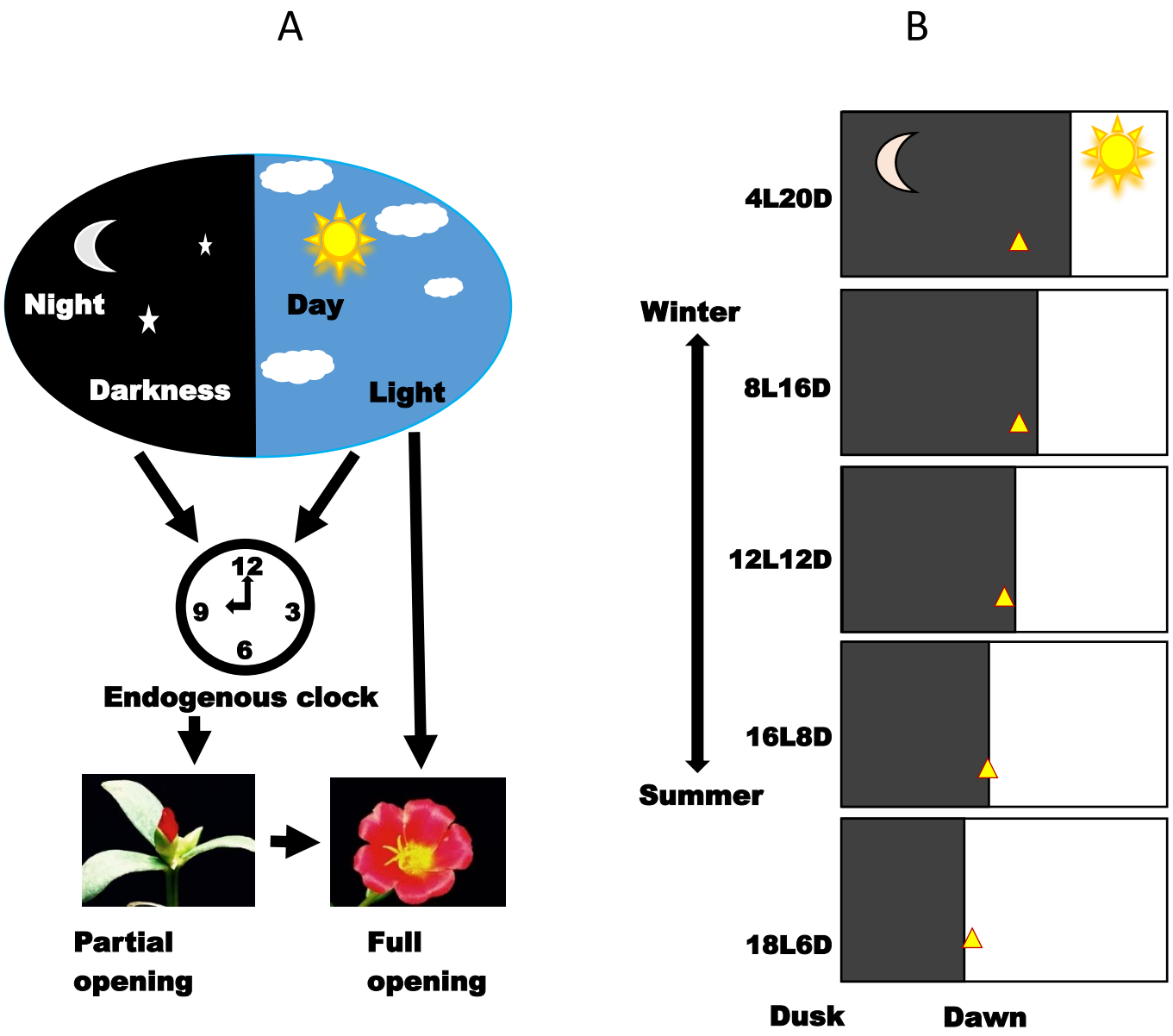

Fig. 4.6 Summary of the role of photoperiods and the circadian clock on flower opening in *P. umbraticola.*

(A) Both light and dark signals are essential for resetting the clock that controls the timing of flower opening. Light is essential for rapid and full opening. **(B)** Longer photoperiods promote earlier flower opening relative to the reference point. Under natural sunlight conditions, as the day becomes longer, the flowers open at an earlier time. Arrows represent predicted time at which the flowers will be at stage 2 of flower opening.

CHAPTER 5

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General Discussion

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In Chapter 3, I revealed the difference in flower longevity between a conventional cultivar 'SR' and a recently produced cultivars 'SCR'. In this study, I managed to show that different factors such as pollination, pistil wounding significantly affects the longevity of both cultivars **(Table 3.3).** The two cultivars used in the study were randomly selected and preliminary investigations show that they both seemed to represent their two groups well (conventional and recently released cultivars). Exogenous ethylene treatments and the use of ethylene action inhibitors demonstrated that the senescence of both cultivars was ethylene dependent **(Table 3.4 and 3.5).** However, the two cultivars showed a significant difference in endogenous ethylene production **(Fig 3.2)**, which explains their observed flower longevity difference **(Table 3.1).** In carnations for example, cultivars with shorter vase life produced higher levels of ethylene than those with longer vase life and the ethylene production correlated with some *ACS* and *ACO* transcripts.

Moreover, I have shown that temperature is an important cue in determining the speed and extend of flower opening **(Table 3.2)** with different cultivars responding differently to temperature below their thresholds. One of the most important factors for consumer satisfaction in the floriculture industry is flower longevity. Knowledge of the factors affecting longevity are crucial in attempts to improve it, it also equips consumers with enough facts of best possible ways to improve vase life.

In Chapter 4, factors affecting the time of flower opening as well as the influence of different light qualities were investigated. *P. umbraticola* consists of ephemeral flowers that open early in the morning in the presence of full light and wilts mid – late afternoon (time

can vary with cultivars and prevailing weather conditions). Once senescence occur the petal will be in – rolled irreversibly and shed off after some time. The *P. umbraticola* plants bear many flower buds which will be at different developing stages and normally during the flowering season, new set of flowers open on a daily basis. The presence of light proved to be very crucial for the speed and extend of flower opening. Flowers opened very slowly in DD and in most cases did not reach the full opening stage **(Fig. 4.2**) Secondly I have shown that besides external cues, endogenous cues are also of paramount importance in determining the time of flower opening in *P. umbraticola;* opening is under the control of a circadian clock which is predominantly observed under continuous darkness **(Fig. 4.3)**. In DD opening occurred synchronously and is initiated at exactly the same circadian time however, in LL the synchronicity was lost and the circadian time of flower opening was unpredictable **(Fig. 4.3 and 4.4).**

General observations have shown that the flowers of *P. umbraticola* open early in summer than other seasons at constant temperatures. This was probably due to the difference in photoperiods between summer and other season, I tested the effect of different photoperiods on the time of flower opening. Using 17 00 h JST as the reference point (all dark/night treatments start at that time), I found that flowers opened early at longer photoperiods than short photoperiods. The time of flower opening was determined by the light on signal, flower opening being strongly synchronous and at least a 6 h darkness was necessary for the synchronicity of individual flower opening **(Fig. 4.5).** Thus the observed earlier flower opening in summer is as a result of longer photoperiods.

It is important to note that, though the light signal determines the time of flower opening, endogenous cues are also of great importance. This is demonstrated by two main facts: opening occurring at the same circadian time when the dark period is at least 6 h and when the photoperiods are long opening starts just before lights on signal. Secondly under short photoperiods opening initiates in the middle of the darkness with the light signal accelerating the process **(Fig. 4.5 and 4.6).** In DD opening occurred at the same circadian time however, LL disrupts the clock function in a manner similar to *elf3* mutants. Thus exogenous cues work with endogenous cues in determining the appropriate time of flower opening. Knowledge of the plants chronobiology in conjunction with the environmental cues is important as these two work together in determining best season for reproduction, they also modulate response to stress, as well as drought tolerance. In attempt to see how far the clock can be manipulated, I entrained the plants to an antiphase photoperiod and I showed that the time of flower opening can be easily manipulated. Flowers of *P. umbraticola* can be entrained to many different photoperiods such that opening time can be at any point of the circadian time (data not shown).

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With the current trend of a global rise in temperatures, *P. umbraticola* provides a realistic adaptation option in the ornamental industry, so basic information on this ornamental plant is promising for promoting the future of the industry. The correlation of ploidy levels and stress tolerance is one area which needs more research so as to deal with the global climactic changes trough breeding of stress tolerance plants. These studies provide a major stepping stone for future improvement of *P. umbraticola* cultivars using modern biotechnology techniques.

Research Summary

In the genus *Portulaca* two species are mainly used for ornamental purpose that is *P. grandiflora* and *P. umbraticola*. The flower shape of *P. umbraticola* resembles that of *P. grandiflora,* and its leaf morphology highly resembles that of common purslane (*P. oleracea*). As a result, *P. umbraticola* cultivars were named as 'Hana-Suberihiyu' in Japanese, which means ornamental *P. oleracea* or were simply called as 'Portulaca', which is its genus name. Ornamental *Portulaca* has recently become an important summer bedding plant in Japan due to its adaptability to the hot and dry weather conditions a typical of the Japanese summer. Recent trends show an increase in the consumers of ornamental *Portulaca,* with *Portulaca* being ranked as the 9th most grown bedding plant in Japan as of 2009. It was estimated that over 70% of the ornamental *Portulaca* would be occupied by *P. umbraticola*. A lot of new *P. umbraticola* cultivars have been recently bred with different flower color, flower diameter and flower longevity. Most of these characteristics associated with the new cultivars have not yet been fully examined, here I report the examination of some of the cultivars with an aim of further developing the breeding of ornamental *Portulaca.*

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Difference in Flower Longevity and Endogenous Ethylene Production of *Portulaca umbraticola Cultivars*

Portulaca umbraticola Kunth, with ephemeral flowers, has become an important summer bedding plant in Japan. A lot of new cultivars have recently been bred with different flowering characteristics, but there is little information about *P. umbraticola* cultivars. In this study, I investigated the differences in flower longevity, endogenous ethylene production and ethylene sensitivity between a conventional cultivar, 'Single Red' (SR), and a newly released cultivar, 'Sanchuraka Cherry Red' (SCR). The flowers of SR opened and closed earlier than those of SCR and the flower longevity of SCR was significantly longer than that of SR. The effects of pollination, filament wounding and pistil removal on flower longevity were also investigated in both cultivars. Pollination, filament wounding and pistil removal significantly

accelerated senescence in both cultivars, but filament wounding was much more significant in accelerating senescence. Endogenous ethylene production from flower opening to closure was significantly higher in SR than in SCR. The peak ethylene production in SR occurred 2 h earlier than that in SCR. Exogenous ethylene treatments of 0.5, 1, and 2 $\mu L \cdot L^{-1}$ significantly accelerated the rate of senescence in both SR and SCR. The use of ethylene action inhibitor 1methylcyclopropene (1-MCP) and ethylene biosynthesis inhibitor aminoethoxyvinylglycine (AVG) significantly improved flower longevity in both cultivars, with the latter being much more effective. The better flower longevity of SCR seems to be related to lower endogenous ethylene production. The senescence of *P. umbraticola* cultivars seems to be ethylene-dependent.

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

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, I used time lapse photography to study the effect of different photoperiods and light qualities on the flower opening rhythm of *P. 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.

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Appendices

Appendix 1. Parameters and websites used for *de novo* transcriptome analysis

Unigene functional annotation

Blast:

version: v2.2.23

parameters: default

website: http://blast.ncbi.nlm.nih.gov/Blast.cgi

Blast2GO:

version: v2.5.0

parameters: default

website: https://www.blast2go.com

InterProScan5:

version: v5.11-51.0

parameters: default

website: https://code.google.com/p/interproscan/wiki/Introduction

Database Information

NT:

description: nucleotide sequence database, with entries from all traditional divisions of GenBank, EMBL, and DDBJ excluding bulk divisions (gss, sts,

pat, est, and htg divisions. wgs entries are also excluded. Not non-redundant.

website: ftp://ftp.ncbi.nlm.nih.gov/blast/db

NR:

description: non-redundant protein squence database with entries from GenPept, Swissprot, PIR, PDF, PDB and NCBI RefSeq

website: ftp://ftp.ncbi.nlm.nih.gov/blast/db

GO:

description: The ***Gene Ontology*** (GO) project is a major bioinformatics initiative to develop a computational representation of our evolving

knowledge of how genes encode biological functions at the molecular, cellular and tissue system levels. Biological systems are so complex that we

need to rely on computers to represent this knowledge.

website: http://geneontology.org

COG:

description: Cluster of Orthologous Groups of proteins, phylogenetic classification of proteins encoded in complete genomes.

website: http://www.ncbi.nlm.nih.gov/COG

KEGG:

description: KEGG (Kyoto Encyclopedia of Genes and Genomes) is a collection of databases dealing with genomes, biological pathways, diseases,

drugs, and chemical substances. KEGG is utilized for bioinformatics research and education, including data analysis in genomics, metagenomics,

metabolomics and other omics studies, modeling and simulation in systems biology, and translational research in drug development.

website: http://www.genome.jp/kegg

SwissProt:

description: UniProtKB/Swiss-Prot is the manually annotated and reviewed section of the UniProt Knowledgebase (UniProtKB).

It is a high quality annotated and non-redundant protein sequence database, which brings together experimental results, computed features and

scientific conclusions.

website: http://ftp.ebi.ac.uk/pub/databases/swissprot

InterPro:

description: InterPro is a resource that provides functional analysis of protein sequences by classifying them into families and predicting the

presence of domains and important sites.

website: http://www.ebi.ac.uk/interpro

Unigene CDS Prediction

Blast:

version: v2.2.23

parameters: default

website: http://blast.ncbi.nlm.nih.gov/Blast.cgi

ESTScan:

version: v3.0.2

parameters: default

website: http://sourceforge.net/projects/estscan

Unigene TF Prediction

getorf:

version: EMBOSS:6.5.7.0

parameters: -minsize 150

website: http://genome.csdb.cn/cgi-bin/emboss/help/getorf

hmmseach:

version: v3.0

parameters: default

website: http://hmmer.org

Unigene Expression

***Bowtie2* :**

version: v2.2.5

parameters: -q --phred64 --sensitive --dpad 0 --gbar 99999999 --mp 1,1 --np 1 --score-min L,0,-0.1 -I 1 -X 1000 --no-mixed --no-discordant -p 1 -k

200

website: http://bowtie-bio.sourceforge.net/ ***Bowtie2*** /index.shtml

***RSEM* :**

version: v1.2.12

parameters: default

website: http://deweylab.biostat.wisc.edu/ ***RSEM***

-Appendix 1-