Thermal Control Suitable for Increasing Petals in *Eustoma grandiflorum* (Raf.) Shinn.

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The number of petals in a flower is one of the most important appearance qualities of ornamental flowers. In *Eustoma*, the number of petals fluctuates significantly and little is known about how it is controlled. We investigated the cultivating conditions that affect the number of petals in double flowers and tried to develop a technique for growing splendid corolla. High temperature in the reproductive phase reduces the number of petals. The transient treatment of high temperature just prior to the petal initiation stage is sufficient to control such a reduction. The measurement of flower bud growth showed that one week of temperature treatment is necessary to control the number of petals in a flower. The integration of our results demonstrated that both daytime and nighttime temperatures affected the number of petals and that the number of petals was clearly correlated with average daily temperature within the range of $20^{\circ}\text{C} < x < 25^{\circ}\text{C}$. This phenomenon applies to various cultivars in *Eustoma grandiflorum*. We propose the greenhouse conditions necessary to achieve both high quality flowers and reduced energy consumption by considering the temperature and stages of flower development.

Key Words: *Eustoma*, greenhouse, petal, temperature.

Introduction

Technological advancements in controlling the environment in greenhouses can lead to higher yield as well as a higher quality product (Adams et al., 2001; Higashide, 2009; Kubota et al., 2012). The installation air conditioning systems and light supplements for agricultural application can efficiently address year-round climate moderation in greenhouses. Therefore, simulation models based on scientific knowledge developed from experiments are useful for agricultural producers to make the most appropriate decisions regarding a given situation. Compared with vegetable plants, for which the environmental manipulation of growth and development have been evaluated (Johkan et al., 2013; Pipattanawong et al., 2009; Takahata and Miura, 2017),

techniques for improving the quality of floral plants have apparently yet to be sufficiently developed, although customers have indicated quality as being most important in a survey on flowers.

Eustoma grandiflorum (lisianthus) originated in the southern part of North America, and is one of the most popular ornamental plants in the world. The appeal of its splendid corolla and variety of colors contributed to increased production in past decades. Along with this trend, *Eustoma* ranks fifth in production value and third in cultivation area among cut flowers in Japan (Ministry of Agriculture, Forestry and Fisheries, http://www.maff.go.jp/j/tokei/kouhyou/sakumotu/sakkyou_kaki/index.html).

The practical cultivation of *Eustoma* began in the 1950s in Japan. At first, all *Eustoma grandiflorum* cultivars as a cut flower were single-type flowers with five petals, the same as wild *Eustoma grandiflorum*. It seems that double flowers, which have multiple whorls of petals per flower, suddenly appeared in the 1960s in Japan (Fukuta, 2016). Given the low initial stability of their phenotype, double flowers were not produced commercially. After breeding of stable double-flower cultivars in the 1980s, double flowers have been produced for commercial purposes. As large numbers of extra petals lead to conspicuous corolla, the production

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of double flowers now significantly exceeds that of single-type flowers. For example, all top 20 varieties of *Eustoma* in terms of production relative to the Japanese market have double flower trait (Japan Flower Promotion Center Foundation, <http://www.jfpc.or.jp/bunseki/2009.html>).

Although the wholesale amount of *Eustoma* in the hot season is much higher than that in the cold season in Japan, the flower quality in summer tends to be lower than that in winter. One reason is that the petal number fluctuates seasonally. Therefore, improvements in cultivation are important regarding the number of petals necessary to provide *Eustoma* with high-end quality.

The phenomenon in which the petal number fluctuates has been observed in other species, such as roses, carnations, and cyclamen (Garrod and Harris, 1974; Ma et al., 2015; Mizunoe and Ozaki, 2015). Double-flower roses mainly result from homeotic conversion from the stamen to petals affected by temperature governing this degree (Dubois et al., 2010; Ma et al., 2015). The fluctuation in petal number coincides with that of the stamen number in cyclamen (Mizunoe and Ozaki, 2015; Mizunoe et al., 2015). In carnations, there are two types of petal number fluctuations: additional petals produced from the secondary floral bud, and increases in petals arising directly from the receptacle (Garrod and Harris, 1974).

In many flowering plants, floral morphogenesis can be explained by the ABC model (Coen and Meyerowitz, 1991). Loss of expression of the *Arabidopsis* C-class gene *AGAMOUS* (*AG*) results in the conversion of stamens and carpels to petals and sepals, respectively and indeterminacy of the floral meristem, leading to double flowers with excess petals. The same mechanism as that of *Arabidopsis* can be adopted in some floricultural plants such as *Ipomea nil*, *Gentiana scabra*, and *Thalictrum thalictroides* (Galimba et al., 2012; Nakatsuka et al., 2015; Nitasaka, 2003). In the case of the double-flowered *Eustoma*, several theories have been postulated about the genetic mechanism. One theory maintains the heterozygous double-flower gene results in a flower with multiple petals, and its homozygous version results in a flower with sepaloid petals (Mato et al., 2004). Another theory postulates that three alleles of a double-flower gene are involved in single-, semi-double, and double-flower phenotypes (Takatori et al., 2015). Regarding the genetic mechanism of double flowers and the biological response to environmental conditions, further studies are needed.

In this study, we showed that inadequate thermal air conditions cause a reduction in the number of petals. We grew *Eustoma* at various air temperatures and clarified the developmental stage at which temperature impacts the petal number. We also demonstrated that we can estimate the number of petals relative to the green-

house temperature. Finally, we developed a technique to produce *Eustoma* with excessive petals using low-cost environmental control in the greenhouse.

Materials and Methods

Plant material and growth conditions

Eustoma grandiflorum ‘King of Orchid (KO)’, ‘Voyage white’, ‘Claris pink (CP)’, ‘Orbe cocktail’, ‘Rosina green’, ‘Amber double purple’, ‘Fours fours violet’ (Sakata Seed Co., Japan), and ‘Paleo pink flash’ (Takii Seed Co., Japan) were planted in plug trays containing fertilized soil and maintained at 10°C in the dark for five weeks in a growth cabinet. Trays were then transferred to a growth chamber (Nihonika Co., Japan) and grown under constant daytime/nighttime preset temperatures of 28°C/18°C and a 12-h photoperiod supplemented with fluorescent lamps (50 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ Photosynthesis Photon Flux Density (PPFD)). Each seedling at the two-pair leaf stage was transplanted to a 10.5-cm plastic pot containing fertilized medium (Kureha Engeibaido; Kureha Chemical Industry Co., Ltd., Japan). In all experiments except for Experiment (Ex.) 5 described below, plants in the vegetative stage were grown in a greenhouse vented at 28°C and heated at 15°C (in case of a nighttime temperature below 15°C). Room humidity was set to 70% when plants were grown in the growth chamber. The length of flower buds was defined as the distance from the bottom end of the sepal to the top of the petal.

In Ex. 1, KO seedlings were transplanted on September 16, 2010. Then, 89 days after transplanting seedlings, we removed floral buds if they were larger than 4 mm. Temperature treatment at 37°C/30°C or 25°C/15°C had been applied to the plants using the growth chamber under a 10-h photoperiod supplemented with fluorescent lamps (400 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ PPFD) until blooming. We counted the petal number of flowers at node 1.

In Ex. 2, KO seedlings were transplanted on May 13, 2011. Temperature treatment at 37°C/27°C or 28°C/18°C was applied to the plants using the growth chamber under a 12-h photoperiod supplemented with fluorescent lamps (400 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ PPFD) from 37 days after transplanting until the flowers opened. Plants subjected to temperature fluctuations (37°C/27°C or 28°C/18°C) every seven days during reproductive phase. If the flower has not bloomed the fifth weeks after temperature treatment, plants keeps under the same temperature as that in fifth week. We removed all floral buds except for those at node 1 and node 2, and compared the petal number of flowers at node 1 and node 2. In Ex. 2, we modified the temperature conditions of Ex. 1 since it seemed slightly stressed and about 8% of samples under both high- and low-temperature conditions resulted in flower-bud blasting.

In Ex. 3, KO seedlings were transplanted on July 22, 2011. Temperature treatment at 37°C/27°C or 20°C/

20°C was applied to the plants using a growth chamber under a 12-h photoperiod supplemented with fluorescent lamps ($400 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ PPFD) from 21 days after transplanting until the flowers opened. The growth stage of flowers during the temperature treatment was determined by paraffin sectioning of plant tips five days after commencing temperature treatment. We evaluated the petal number when flowers opened.

In Ex. 4, KO seedlings were transplanted on September 22, 2011. After 73 days of growth in a greenhouse, temperature treatment at 28°C/18°C was applied for three days to plants using the growth chamber under a 12-h photoperiod supplemented with fluorescent lamps ($190 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ PPFD) for acclimation. Then the plants were grown in one of four preset temperature environments: 35°C/25°C, 35°C/15°C, 25°C/25°C, or 25°C/15°C under a 12-h photoperiod supplemented with fluorescent lamps ($190 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ PPFD) for two weeks. After temperature treatment, we measured the length of all floral buds and removed them except for 2 floral buds per plant, which were on the main stem and almost similar sizes. All plants were grown under 28°C/18°C using the growth chamber under a 12-h photoperiod supplemented with fluorescent lamps ($190 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ PPFD) until the flowers opened. We counted and compared the petal number of groups, of which the floral bud sizes were not significantly different.

In Ex. 5, KO seedlings were transplanted on August 21, 2013 and grown in the growth chamber under constant daytime/nighttime temperatures of 28°C/18°C under a 14-h photoperiod supplemented with fluorescent lamps ($190 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ PPFD) for 28 days. Thereafter, the plants were exposed to one of eight environments where the daytime was under a 12-h photoperiod supplemented with fluorescent lamps ($190 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ PPFD) and the nighttime temperature was 15°C. Then 29 days later, we measured the floral buds at node 1 and node 2 and removed buds except for them. The plants were placed under the conditions of the growth chamber at 28°C/18°C under a 14-h photoperiod supplemented with fluorescent lamps ($190 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ PPFD) until the flowers at node 2 opened. We counted the petal number of flowers at node 2.

In Ex. 6, KO seedlings were transplanted on July 22, 2011 (Ex. 6-1) and March 26, 2015 (Ex. 6-2). In Ex. 6-1, Temperature treatment at 28°C/18°C or 20°C/20°C was applied to the plants in the growth chamber under a 12-h photoperiod supplemented with fluorescent lamps ($400 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ PPFD) from 21 days after transplanting until the flowers opened (28°C/18°C: September 20, 20°C/20°C: September 30 on average). In Ex. 6-2, all plants were grown in a greenhouse vented at 28°C until the flowers opened (June 23, 2015 on average). Flower bud length of both node 1 and node 2 were measured every seven days over a five-week

period. We tested 6 plants in Ex. 6-1 and 10 plants in Ex. 6-2.

In Ex. 7, five *Eustoma* cultivars of different colors and shapes were planted and grown in the greenhouse vented at 28°C for 41 days. Starting on July 30, 2015, we removed floral buds if they were larger than 4 mm. The plants were grown in one of two environments: in the greenhouse or in the growth cabinet at constant temperature of 20°C under a 12-h photoperiod supplemented with fluorescent lamps ($400 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ PPFD). Then 19 days later, the plants were carried back to the greenhouse. The max/min temperature during 19 days in the greenhouse was 43.8°C/23.1°C. We counted the number of petals of 3 floral buds, which were at node 2 on main stem and at node 1 and node 2 on the axillary branch below the main stem.

In Ex. 8, KO seedlings were transplanted on December 11, 2015. After cultivation for 58 days in the greenhouse, the plants were grown in one of three environments: 25°C/25°C, 25°C/20°C, or 25°C/15°C under a 12-h photoperiod supplemented with fluorescent lamps ($190 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ PPFD) for 45 days. After temperature treatment, all plants were grown in the greenhouse vented at 28°C until the flowers of node 3 opened.

In Ex. 9, KO seedlings were transplanted on April 18, 2016. After 36 days (on May 24), the plants started growing in one of four environments: (1) no temperature control (maximum temperature; 34.2°C, minimum temperature; 14.8°C), (2) low-temperature control at night (6:00 PM–6:00 AM, setting temperature: 16°C), (3) during the daytime (9:00 AM–6:00 PM, setting temperature: 20°C), (4) low-temperature control from evening to morning (4:00 PM–9:00 AM, setting temperature: 16°C) under natural light condition and with heat pump system (CS-283CF-C; Panasonic, Co., Japan), remote controller (RC-32AC; ELPA Co., Japan), and partition curtain (Ace III; Seiwa, Co., Japan). We corrected the initial setting mistake of low-temperature control in (4) on May 30, in which the room was cooled not at early morning (6:00 AM–9:00 AM) but evening and night (4:00 PM–6:00 AM) (Fig. S1). After temperature treatment for 21 days, all plants were grown in a greenhouse vented at 28°C until the flowers opened.

Histological analysis

For paraffin sectioning, we collected floral bud samples and fixed them overnight in formalin/glacial acetic acid: 70% ethanol; 1:1:18, followed by dehydration in a graded ethanol series. Following substitution with xylene, the samples were embedded in Paraplast Plus (Oxford Labware, St. Louis, USA) and sectioned to a thickness of 8 μm using a rotary microtome. Sections were stained with 0.05% toluidine blue and observed with a light microscope.

Results

Evaluation of effective floral organ identity and effective environment on petal number (Ex. 1)

The double flower of *Eustoma* has five sepals, several petal whorls with five petals each, five stamens, and one pistil (Fig. 1A, B). Petal-stamen intermediate organs are sometimes formed. The double flowers of *Eustoma* show no homeotic conversion from sepals

Fig. 1. Evaluation of temperature on the floral organ number in 'KO'. (A, B) Seasonal differences in flower appearance. A flower grown in the hot season (A) as photographed in September, and a flower grown in the cold season (B) photographed in January. (C) A flower grown under high (left, day-time temperature/nighttime temperatures; 37°C/30°C) and low (right, 25°C/15°C) temperature conditions. (D, E) Effects of ambient temperature conditions in the reproductive phase on the floral organ number (D) and the bloom timing (E) in Ex. 1. In (E), scores show the period from commencing temperature treatment to bloom. Vertical bars indicate $\pm$ SD. ($n = 33$ in the 37°C/30°C treatment group and $n = 19$ in the 25°C/15°C treatment group). *** indicates significance at the 0.1 % level ($P < 0.001$) by *t*-tests.

and/or stamens into petals, so that the numbers of floral organs except for double-flower petals and petal-stamen intermediate organs are the same as in single flowers.

The petal number fluctuated seasonally. For example, a flower that bloomed in January (min 13.4°C–max 22.25°C) tends to have more petals than those in September (min 13.4°C–max 40.2°C) (Fig. 1A, B), suggesting that ambient temperature affects the number of petals in *Eustoma*. To confirm this effect, we investigated the petal number of plants grown in the growth chamber under different air temperatures. Since cold temperature delays the flowering of *Eustoma* (Zaccari and Edri, 2002), we changed the temperature when the reproductive phase began. That is, we evaluated the effects of temperature in the reproductive phase. The flowers under high-temperature treatment at 37°C/30°C had fewer petals than those under low-temperature treatment at 25°C/15°C (Fig. 1C, D), which suggests that the number of petals is associated with temperature in the reproductive phase. With respect to organ identity, the petal was the only floral organ affected by temperature (Fig. 1D). Therefore, low-temperature treatment increased the total number of floral organs of a flower. Low-temperature treatment in the reproductive phase resulted in 16 days delay of blooming (Fig. 1E).

Analysis of effective developmental stage on petal number (Ex. 2)

To identify the developmental stage sensitive to a high-temperature-induced reduction of the petal number in *Eustoma*, short-term temperature treatment was applied to plants in the reproductive phase. A test section 13 was provided in which the weekly shift pattern of temperature was used (Fig. 2A). The petal number of flowers at node 1 was not significantly different among treatment groups (Fig. S1). The petal number of flowers at node 2 under low temperature at 28°C/18°C was 17.1 ± 2.7 , while that under high temperature at 37°C/27°C throughout the reproductive phase was 9.1 ± 1.6 (Fig. 2B, groups F and G). Transient high-temperature treatment in the first week decreased the petal number most significantly (Fig. 2B, groups A–F). The first week was also the best timing for transient low-temperature treatment to increase the petal number (Fig. 2B, groups G–L). Therefore, flower buds were most sensitive to the temperature of the first week. Moreover, prolonged temperature treatment resulted in the same effect as one-week treatment (Fig. 2B, groups B–F, H, and M), indicating that about one week is necessary and sufficient period for petal number control.

There was a difference of 7.5 days in the blooming time between high- and low-temperature treatments for 5 weeks (Fig. 2C, groups F and G). In contrast, a transient temperature change for 1 week cause no difference in the timing of bloom among groups A–F and groups G–L, respectively (Fig. 2C).

Fig. 2. Effects of transient temperature treatment on the petal number in Ex. 2. (A) Schematic diagrams show developmental stages and temperature conditions. Gray and white color coded blocks represents high (37°C/27°C) and low (28°C/18°C) temperature conditions, respectively. Arrow shows the sequence of developmental stages in reproductive phase. (B, C) Effects of temperature treatment on the petal number (B) and on the timing of bloom (C). Scores in (C) show the period from commencing temperature treatment to bloom of flowers at node 1. Different letters denote significant differences among treatments at $P < 0.05$ by Tukey-Kramer multiple comparison tests. Vertical bars indicate $\pm$ SD ($n = 9$ in treatment group F and group G. $n = 6$ in group A to E and H to M).

Identification of developmental stage sensitive to temperature (Ex. 3)

We previously described the initial developmental process of *Eustoma* flowers (Kawakatsu et al., 2012). In order to identify the specific developmental stage that is sensitive to ambient temperature relative to controlling the petal number, we examined the developmental stage of floral buds five days after temperature treatment by paraffin sectioning. In this experiment, the flower at node 2 under low-temperature treatment had a much larger number of petals than that without treatment (Fig. 3A, B). In contrast, temperature treatment did not affect the petal number of the flower at node 1. These results may reflect the developmental stage of flowers with acute temperature treatments. We previously analyzed the branching patterns in *Eustoma* (Kawakatsu and Fukuta, 2012). Each floral developmental stage differs depending on its node because the inflorescence meristem and floral meristem differentiate synchronously.

In both high- and low-temperature treatment groups, the floral buds at node 1 were at the developmental stage of the second/ third petal or stamen initiation (Fig. 3C). In both high- and low-temperature treatment

groups, the floral buds at node 2 were at the stage of first petal or sepal initiation or earlier. These results revealed that the stages sensitive to temperature affecting the petal number are those before the petal differentiation stage.

Relationship between the petal number and daytime/nighttime temperatures (Ex. 4)

To gain insight into the index of temperature control, we grew plants in one of four environments: 35°C/25°C, 35°C/15°C, 25°C/25°C, or 25°C/15°C. We compared the petal number of flowers whose length at the end of temperature treatments were almost the same (Fig. 4A). The plants under 25°C/15°C temperature had 26.4 petals—the most among the four groups. The most effective 10°C decrease in temperature occurred in the daytime from 35°C to 25°C in the case of a nighttime temperature of 15°C, which resulted in 15.1 more petals (Fig. 4B, group 2 vs group 4). In contrast, a 10°C decrease in the daytime from 35°C to 25°C in the case of a nighttime temperature of 25°C had no effect on the petal number (group 1 vs group 3). As for temperature treatment at night, a 10°C decrease at night from 25°C to 15°C in the case of a daytime temperature of 35°C had no effect on the petal number (group 1 vs group 2). In contrast, a 10°C decrease at night from 25°C to 15°C in the case of a daytime temperature of 25°C resulted in an increase of more than 10 petals (group 3 vs group 4). Taken together, both daytime and nighttime temperatures seem to affect the petal number, but it changes in the specified temperature change.

Relationship between the petal number and daily mean temperature/daily maximum temperature (Ex. 5)

To further obtain a detailed index of temperature control in greenhouses, we investigated the relationship between the petal number and daily mean temperature/daily maximum temperature, respectively. We set temperature condition to that of a winter greenhouse, in which the windows are kept shut to prevent the loss of treated CO₂ and temperature sometimes exceeds 30°C in the daytime. Figure 5A shows the temperature condition. We confirmed that the size of the flower buds at the end of temperature treatment, at which point the temperature-sensitive stage had passed. Plants continuously under 25°C daytime temperature for 29 days had 23.3 ± 6.8 petals, and plants continuously under 33°C daytime temperature had 11.5 ± 2.5 petals on average (Fig. 5A, group 1 and group 8). When we compared petal numbers among the different groups having the same daily mean temperature, the groups with different periods of 33°C exposure in the daytime had almost the same number of petals (i.e. group 1, group 3, group 5, and group 7).

Finally, the detection data in Ex. 4 and Ex. 5 showed that daily mean temperature and petal number were correlated within the range of $20^\circ\text{C} < x < 25^\circ\text{C}$ (Fig. 5B).

Fig. 3. Identification of developmental stage in which temperature affects the petal number. (A) A schematic diagram of inflorescence in *Eustoma*. An arrow indicates an axillary branch. (B) The number of floral organs in Ex. 3. Vertical bars indicate $\pm$ SD of the petal number ($n = 5$). Intermediate organ means intermediate organ between petal and stamen. (C) The floral developmental stages of plants five days after commencing temperature treatment ($n = 3$). (D) A paraffin section of floral buds grown under 20°C/20°C. Scale bar = 1 mm. Abbreviations; se: sepal; pe: petal; br: bract.

Fig. 4. Effects of day and night temperature on the petal number in Ex. 4. (A) The size of floral buds at the end of temperature treatments. Not significantly different among the samples (one-way ANOVA). Vertical bars indicate $\pm$ SD ($n = 6-7$). (B) The petal number. Different letters denote significant differences among treatments at $P < 0.05$ by Tukey-Kramer multiple comparison tests. Vertical bars indicate $\pm$ SD ($n = 6-7$).

Even in the 'Voyage white', the same relationship between average temperature and petal number was correlated within the range of 20°C $< x < 25$ °C as in the 'KO' (data not shown).

Estimation of the period required for thermal control by comparing the growth curves of flowers (Ex. 6)

The critical stage for petal number determination is when the floral buds measure less than 2 mm (Fig. 3C, D), and are almost totally covered and hidden by bracts. In order to determine when low-temperature treatment should be applied, clarifying the degree of separation between the developmental stage of one flower from that of the flower in the lower node could prove useful. To clarify this, we continuously measured the floral bud length and calculated the difference in growth between both nodes from the approximated growth curve in Ex. 6-1 (Fig. 6A, B). For example, the growth curve of the floral bud at node 2 grown under 28°C/18°C in the growth chamber showed $y = 1.4368e^{0.0798x}$ (y : floral bud length, x : days after treatment), and the growth curve of the floral bud at node 1 was approximately equal to the graph in which x is shifted for 6 (Fig. 6B). When plants were under 20°C/20°C ambient temperature, the growth pattern of floral buds at node 1 deviated at about 7 days from that at node 2 (data not shown). We also confirmed there was about a 5.5 day deviation of flower development between node 1 and node 2 grown in a greenhouse in Ex. 6-2, in which day/night temperature during the differentiation stage of floral organs was 25.3°C/20.8°C on average (data not shown).

Adaptability test of Eustoma cultivars (Ex. 7)

To verify whether our results were applicable to various cultivars of *Eustoma*, we transiently exposed the plants of five cultivars to artificial conditions of lower temperature and light intensity than that in a greenhouse. The environmental change applied during the

Fig. 5. Effects of exposure duration to daily maximum temperature and daily mean temperature on the petal number. (A) Schematic diagram of the temperature condition in Ex. 5 (left) and the number of petals (right). Different letters denote significant differences among treatments at $P < 0.05$ by Tukey-Kramer multiple comparison tests. Vertical bars indicate $\pm$ SD ($n = 3-10$). (B) Relationship between the number of petals and daily mean temperature, derived from the results of Ex. 4 and Ex. 5.

Fig. 6. Developmental differences in floral buds between node 1 and node 2. (A) Changes in floral bud length when grown under 28°C/18°C. Lighter-colored solid line shows the approximate curve of floral buds at node 1; lighter-colored dotted line shows the approximate curve of floral buds at node 2. (B) Relationship between the growth curve of floral buds at node 1 and the graph as it deviated six days from the approximate growth curve of floral buds at node 2. Bold-colored solid line: changes in floral bud length at node 1, Lighter-colored solid line: approximate curve of floral buds at node 1 ($y = 1.5007e^{0.0923x}$). Bold colored dotted line: graph as it deviated six days from the approximate growth curve of floral bud at node 2.

early flower differentiation stage induced an increased number of petals for each cultivar tested (Fig. 7A) and coincided with the results of experiments with 'KO',

Fig. 7. Adaptability of thermal control treatment to increase petals. (A, B) The effects of low temperature treatments on the petal number (A) and the blooming time (B). Scores in (B) show the period from commencing temperature treatment to bloom of flowers at node 1. Vertical bars indicate $\pm$ SD ($n = 3-8$). ** and *** denote significant at $P < 0.01$ and 0.001 , with Welch's test, respectively.

thereby showing that this phenomenon is common among various cultivars of *Eustoma*. Low-temperature treatments resulted in a bloom delay of more than 7 days (Fig. 7B).

Cooling nighttime temperature increases petal numbers under both artificial light and natural light conditions (Exs. 8 and 9)

Low daytime temperature resulted in an increased number of petals (Fig. 5). Next, we examined whether low nighttime temperature could lead to an increased number of petals. In our experiment involving a growth chamber, low-temperature treatment at night increased the number of petals depending on the temperature (Fig. 8A). Temperature treatment at 15°C for 45 days was enough to bear flowers with an increased number of petals at all three nodes. This, however, caused about 1 week later harvesting (Fig. 8B).

Finally, we evaluated the effect of temperature in a greenhouse. Under natural light conditions, cooling with a heat pump system significantly influenced the mean temperature and improved the petal number in both 'KO' and 'CP' (Table 1; Figs. 9A–C, S2). Furthermore, temperature treatment for 21 days prevented the delay in blooming (Table 1). With respect to the harvest time, cooling during 4:00 PM–9:00 AM led to the delay of about 3 days (data not shown). These results demonstrated that low-temperature treatment at any time of the day can lead to an increase in petals, while short-term cooling had little effect on extending the cultivation period.

Fig. 8. Effects of nighttime temperature on the petal number under artificial light conditions. (A) The number of petals at each node in Ex. 8. Data are the means $\pm$ SD ($n = 10$ – 12). (B) Period from commencing temperature treatment to harvest. Significant difference based on Tukey-Kramer multiple range test ($P < 0.05$) is indicated by different letters.

Discussion

Environmental conditions for increasing the number of petals

The purpose of this study was to develop a technique for high-quality flower cultivation in *Eustoma*. Inadequate thermal conditions such as an above 25°C daily mean temperature resulted in a lower petal number. This phenomenon affected various varieties of *Eustoma* (Fig. 7) and caused low flower quality in the hot season. The results of Ex. 5 suggest that the petal number apparently correlates not with the daily maximum temperature, but with the daily mean temperature (Fig. 5). The detection data in Ex. 4 and Ex. 5 showed that daily mean temperature and petal number were correlated, especially clearly within the range of $20^{\circ}\text{C} < x < 25^{\circ}\text{C}$ (Fig. 5B), suggesting that we should prevent the initiation stage of floral buds corresponding with the hottest season, in which mean air temperature tends to exceed 25°C.

It was demonstrated that a low temperature condition in the daytime is relatively inappropriate for the photosynthesis influencing the growth rate in *Eustoma* (Sato et al., 2001; Trudgill et al., 2005). Our previous study also demonstrated CO₂ enrichment resulted in promoting growth in the cold season, which is characterized by a short period of daylight and low temperature (Ushio et al., 2014). Hence, the daytime temperature in a greenhouse in the cold season tends to exceed 28°C, due to keeping windows shut to prevent the loss of CO₂ and heat. This raises concern over the decreased number of petals in doubled flowers caused by elevated temperature even in the cold season. Our results demonstrated that a prolonged period of plant exposure to elevated temperature does not always decrease the number of petals, provided that the average temperature is properly set (Fig. 5A).

For commercial growers, the cost of cooling a greenhouse represents a significant production cost. Although low daytime temperature results in an increased number of petals (Figs. 1–5, 7), the cost of greenhouse cooling in the daytime is higher than that at night. Therefore, we examined whether low nighttime temperature using a heat pump could lead to an increased number of petals. Low-temperature treatment at night with artificial light facilitated an increase in the number

Table 1. Temperature condition in a greenhouse and effects on the blooming time in Ex. 9.

Temperature condition		Control	Cooling 6:00 PM–6:00 AM	Cooling 9:00 AM–6:00 PM	Cooling 4:00 PM–9:00 AM
Mean air temperature (°C)		23.9	19.7	21.0	18.3
Period from commencing treatment to bloom at node 1	KO ^z	41.2 $\pm$ 2.8	42.4 $\pm$ 2.4	43.5 $\pm$ 3.1	42.9 $\pm$ 2.1
	CP ^z	36.9 $\pm$ 3.0	40.4 $\pm$ 0.7	40.9 $\pm$ 1.9	38.7 $\pm$ 3.2

Data are the means $\pm$ SD (KO: $n = 16$, CP: $n = 7$).

^z not significantly different among the samples (one-way ANOVA).

Fig. 9. Flower cultivation with temperature control to increase petals under natural light condition. (A) Temperatures at 10 min intervals for 4 days during Ex. 9. The colors of graphs are Control (blue), Nighttime cooling (red), Daytime cooling (green), Morning & evening & Nighttime cooling (purple). (B) The number of petals at each node in Ex. 9. Data are the means $\pm$ SD ($n = 16$ in 'KO', $n = 7$ in 'CP'). The colors of graphs in (B) are corresponding to those in (A). Significant difference based on Tukey-Kramer multiple range test ($P < 0.05$) is indicated by different letters. Z indicates there is no significantly difference among the samples (one-way ANOVA). (C) Appearance of 'KO' flowers at node 3 grown under different daytime/nighttime temperatures in greenhouses in Ex. 9. Left to Right: Control, Nighttime cooling (6:00 PM–6:00 AM), Daytime cooling (9:00 AM–6:00 PM), and Morning & evening & Nighttime cooling (4:00 PM–9:00 AM).

of petals (Fig. 8). Finally, we managed to produce *Eustoma* with more petals using heat pump cooling in the greenhouse (Fig. 9). Therefore, cultivation controlling the mean daily temperature could bring about flowers with an increased number of petals. Evaporating cooling systems such as a fan-pad system and a mist system have recently been used for the practical cooling of greenhouses in the daytime during the hot season (Ganguly and Ghosh, 2007). A heat pump air condition-

ing system also enables the benefits of daytime and nighttime temperature control. Combining these systems will achieve both a high growth rate and an increased number of petals in double flowered *Eustoma*, even in the extremely hot season.

Daytime temperature exceeding 30°C is beneficial for the growth for *Eustoma*, given its rapid growth and early flowering (Tsukada et al., 1982; Zaccari and Edri, 2002), and also promotes the opening of flowers (Kudo

et al., 2012). However, the high temperature condition is not good for generating many petals in double flowered *Eustoma*. Therefore, the period of low-temperature exposure to increase the number of petals should be as short as possible. Considering efficient low-cost environmental control, it is important to understand how the petal number is temporally controlled.

Effective and economical temperature treatment

Histological analysis showed that the temperature-sensitive stage for petal number alteration begins at least one week before the petal differentiation stage (Fig. 3C, D). Although floral buds are hardly visible from outside during the sensitive stage, we can estimate them by comparing the stages of previous flowers (Fig. 6). We had previously collected initial floral buds for paraffin sectioning every five days, and confirmed the relationship between developmental stages and flower bud length (Kawakatsu et al., 2012). In addition, we continuously measured the floral bud length (Fig. 6). Therefore, the proper timing of low-temperature treatment could be referenced based on such data. In other words, the temperature for one week before the floral buds at node 1 become about 3 mm affects the number of petals of flowers at node 2. This supposition is supported by our results that the length of flower buds at the end of temperature treatment in Ex. 4 was about 4 mm and that the petal number of flowers was influenced by the low-temperature treatment for 2 weeks (Fig. 4B).

As for pinch, removing the terminal growing flower, our data will be helpful in deciding the timing of low-temperature induction. In other words, low-temperature treatment just after a floral bud at node 1 becomes visible with turning over bracts increased the number of petals of flowers at node 2 and those at the axillary buds below node 1 (Fig. 10). The pinch of a flower at node 1, which becomes big enough to remove easily in 10 days, could promote the growth of axillary buds. Af-

terwards, we should stop the cooling after confirming the floral buds sizes are adequate. Finally, we obtain high-end quality *Eustoma* composed of multi branches with flowers with an increased number of petals.

Our experiments showed that low-temperature treatment both during the daytime and nighttime throughout the reproductive phase caused over two weeks' delay in blooming (Fig. 1E). In contrast, nighttime cooling through the reproductive phase for 45 days under artificial lights resulted in less than 7 days' delay in harvesting (Fig. 8B). Furthermore, short-term low-temperature treatment during daytime and/or nighttime under natural light conditions caused no delay of blooming (Fig. 2C; Table 1). Referring to these results, agricultural producers should be able to significantly reduce energy consumption, while still maintaining an abundant number of petals.

Comparison of doubled flower among various ornamental plants

In double-flowered *Eustoma*, there is no homeotic conversion from stamens into petals, suggesting that it is not caused by a loss-of-function mutation in the class C gene, although the expression pattern of class C genes of *Eustoma* follow the ABC model of *Arabidopsis* (Ishimori and Kawabata, 2014). The fluctuation in the petal number due to temperature has been observed in other species, such as roses, carnations, and cyclamen (Garrod and Harris, 1974; Ma et al., 2015; Mizunoe and Ozaki, 2015). Double-flower roses and cyclamen mainly result of homeotic conversion from the stamen to petals affected by temperature (Dubois et al., 2010; Ma et al., 2015; Mizunoe and Ozaki, 2015; Mizunoe et al., 2015). In carnations, high-temperature treatment elevates the number of petals (Garrod and Harris, 1974). In *Eustoma*, the petal is the only floral organ whose number was influenced by temperature. From a perspective based on organ identity, the nature of the double flower in *Eustoma* is the more similar to that in carnations than that in roses and cyclamen. Regarding the response to temperature, however, the behavior of *Eustoma* may not be exactly the same as that in carnations.

Our results that a linear relationship exists between temperature and petal development (Fig. 5) suggest that some properties affecting the kinetic rate could be involved in the increase in petal numbers. Regarding the promotion of stamen petaloidy in roses, epigenetic DNA methylation appears to contribute to the ambient temperature modulation of RhAG expression, a rose homolog of the *Arabidopsis thaliana* AGAMOUS C-function gene (Ma et al., 2015). Further identification of genes inducing excessive petals will help us to understand how the petal number is controlled in ornamental crops, although the mechanisms involving double flowers may be different depending on the plant species.

Fig. 10. Schematic diagrams of the temperature treatment used to increase the number of petals.

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