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Energy-saving Seedling Production System for Super-forcing Cultivation of June-bearing Commercial Strawberry

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Abstract. **Previously, we showed that a reduction of *FaTFL2* (homolog of *Arabidopsis thaliana TFL1*) expression is a key signal for flowering in domesticated octaploid strawberries (*Fragaria ×ananassa*). Since *FaTFL2* expression seemed to be regulated by temperature rather than by photoperiod, we investigated whether down regulation of *FaTFL2* and floral meristem generation occurred at different temperature conditions. In addition to the conditions for a normal super-forcing cultivation system of an 8-hour photoperiod and day/night temperatures of 31.2 or 30/15 °C, flowering also was generated under the same photoperiod and day/night temperatures of day/night-half/night-half temperatures of 30/15/25 °C conditions. We demonstrate that the new super-forcing cultivation system is energy saving based on the reduction of *FaTFL2* expression.**

June-bearing commercial strawberries (*Fragaria ×ananassa*) are preferable to everbearing ones for Japanese consumers due to their high quality. The June-bearing strawberry is typically a short day and low temperature (SDLT) type plant, and its harvesting time under natural conditions is from March to May or from November to May under a heating system during the winter. Autumn is the off-season, however, not only for consumers, but also confectionery makers who desire the June-bearing type in autumn. SDLT types can initiate flowering under 15 °C (or 17 °C) regardless of the photoperiod conditions, but inhibition of flowering occurs at high temperature (25 to 26 °C) conditions even under a short photoperiod (Bradford et al., 2010; Taylor, 2002; Verheul et al., 2006).

A super-forcing cultivation system has been developed in Japan for producing SDLT type strawberries from September to November (Yamasaki et al., 2003). The SDLT plants are grown in the field during daytime (average 31.2 °C), but they are placed into a curtained tunnel tent with air cooling (15 °C) from 1700 HR to 900 HR for 30–35 d to induce flower buds (Nakajima et al., 2014). As the system consumes a lot of energy, energy saving technology is strongly desired in Japan, especially after the Great East Japan Earthquake on 11 Mar. 2011.

Previously, we clarified that a reduction of *FaTFL2* expression is a key signal for flowering in SDLT type commercial strawberries, and its expression seems to be regulated by temperature rather than photoperiod (Nakajima et al., 2014). In this study, we showed the reduction of *FaTFL2* expression could be used as an indicator for flower initiation, and also demonstrated a new super-forcing cultivation system under a higher temperature condition than the previous condition, which leads to energy saving.

Materials and Methods

Plants and cultivation. Runner plants of *Fragaria ×ananassa* cv. Tochiotome strawberry having three expanded leaves were cut off and planted in pots. We removed the leaves from the plants with the exception of three to five sets of the youngest fully developed leaves. For analyzing the temperature effect on strawberry flowering, we placed the 'Tochiotome' plants in a growth chamber under expected flower noninductive short day and high temperature conditions (SDHT 30/25; an 8-h light at 30 °C and a 16-h dark at 25 °C, used as a negative control), short day and middle temperature conditions (SDMT 30/20; an 8-h light at 30 °C and a 16-h dark at 20 °C), flower inductive SDLT conditions (SDLT 30/15; an 8-h light at 30 °C and a 16-h dark at 15 °C, used as a positive control), and short day and half low temperature conditions (SDHLT 30/15/25; an 8-h light at 30 °C, an 8-h dark at 15 °C, and an 8-h dark at 25 °C) to find the correlation between *FaTFL2* expressions and flower bud formation (Fig. 1). We sampled the crowns of 'Tochiotome' plants on 0, 20, 25, 30, and 35th day after the SDHT, SDMT, SDLT, and SDHLT treatments. Tissues were stored at –80 °C.

Flower buds differentiation. We investigated the developmental stages of the flower buds on samples of SDHT 31.2/15 (an 8-h light at 31.2 °C average/a 16-h dark at 15 °C) at 2013, and those of SDHT 30/25, SDMT 30/20, SDLT 30/15, and SDHLT 30/15/25 at 2014 by microscope. The developmental stages of the flower buds were defined as: stage 0—an undifferentiated stage, stage A1-A3—an initial to late differentiation stage, stage B1-B3—an initial to late flower cluster formation stage, stage C—an initial sepal formation stage, and stage D—an involucre formation stage.

RNA extraction. Total RNA was isolated using the hot borate method described in Wan and Wilkins (1994) with some modifications. A detailed protocol is in our previous paper (Nakajima et al., 2014).

Quantitative real-time polymerase chain reaction (qPCR) analyses of FaTFL1–1 and FaTFL2 genes. Quantification of transcripts for *FaTFL1–1* and *FaTFL2* was conducted using the SYBR Premix Ex Taq II (Tli RNaseH Plus; TaKaRa Bio Inc., Shiga, Japan) and the thermal cycler Dice Real Time

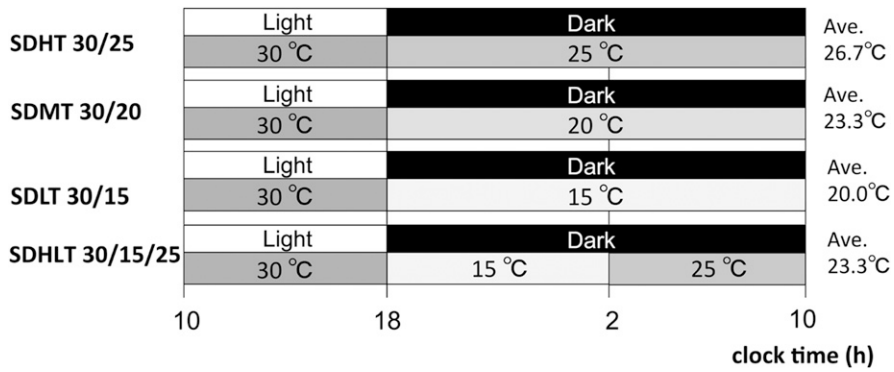

Fig. 1. Different temperature conditions of *Fragaria ×ananassa* Tochiotome for super-forcing cultivation. Short day and high temperature (SDHT) 30/25; SDHT (an 8-h light at 30 °C and 16-h dark at 25 °C, negative control), short day and middle temperature (SDMT) 30/20; short day and low temperature (SDLT) (an 8-h light at 30 °C and 16-h dark at 20 °C), SDLT 30/15; an 8-h light at 30 °C and 16-h dark at 15 °C), short day and half low temperature (SDHLT) 30/15/25 (an 8-h light at 30 °C, 8-h dark at 15 °C and 8-h dark at 25 °C). All 'Tochiotome' plants were grown in a growth chamber.

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System TP800 (TaKaRa Bio Inc.). *MSI1* was used as housekeeping gene (Mouhu et al., 2009). Complementary DNA was synthesized using a *PrimeScript* reverse transcriptase reagent kit with genomic DNA eraser (TaKaRa Bio Inc.). The primer sets are listed in Table 1. qPCR conditions were preheating for 30 s at 95 °C, followed by 45 cycles of 5 s at 95 °C, 10 s at 60 °C, and 25 s (for *FaTFL1–1*) or 20 s (for *FaTFL2*) at 72 °C. The data were analyzed with a Thermal cycler Dice Real-Time System Software Ver. 3.00D (TaKaRa Bio Inc.). The relative expression of each sample was normalized to the *MSI1* gene. The expression of *FaTFL1–1* or *FaTFL2* in three biological replicates at 0 d was used as a reference and was set as 1-fold. qPCR expression analysis was performed using three independent RNA preparations (three biological replicates) per stage, and data are shown as mean ± SE.

Table 1. Primer sets for quantitative real-time polymerase chain reaction analyses.

Primer	Oligonucleotides (5′→3′)	Reference
FaTFL1F	CACCTCGACTGGATTGTGAC	This study
FaTFL1R	TTTGGGATCTGGCCTGCCTC	This study
FaTFL2F	TGACAGTGACTTACAACTCC	This study
FaTFL2R	CCTCCCTTCCAAATGTGTTG	This study
MSI1F	TCTCCACACCTTTGATTGCCA	Mouhu et al. (2009)
MSI1R	ACACCATCAGTCTCCTGCCAAG	Mouhu et al. (2009)

Results and Discussion

In the super-forcing cultivation system for June-bearing commercial strawberry, the plants are grown under SDLT conditions for 30 to 35 d (SDLT 31.2/15; an 8-h photoperiod from 0900 HR to 1700 HR, average 31.2 °C at outside /a 16-h dark from 1700 HR to 900 HR,

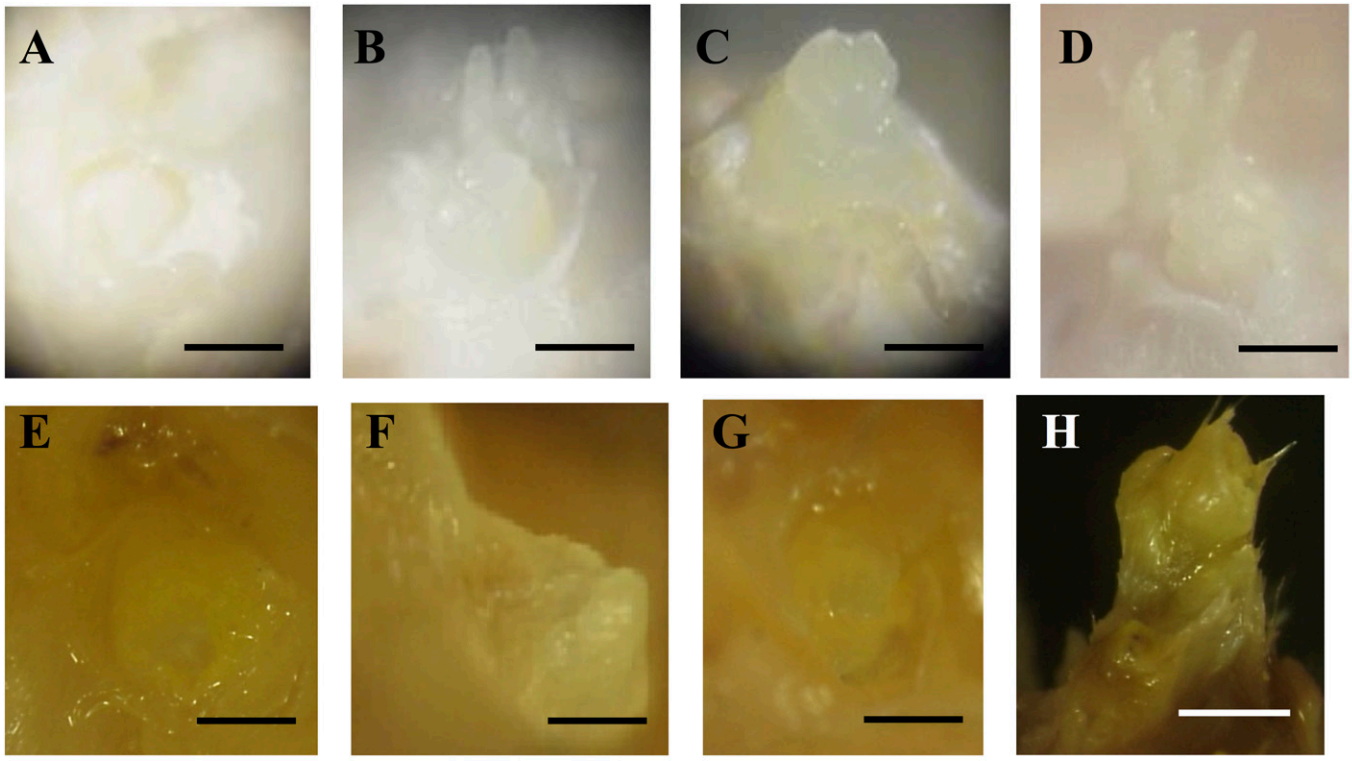

Fig. 2. Flower bud differentiation process of *Fragaria ×ananassa* Tochiotome. (**A**) 25 d after short day and low temperature (SDLT) 31.2/15 (SDLT condition, 8-h light at 31.2 °C average/16-h dark at 15 °C) treatment. A growing point is flat and undifferentiated (stage 0). (**B**) 30 d after SDLT 31.2/15 treatment. A growing point is roundish and upheaves (stage A3). (**C**) 35 d after SDLT 31.2/15 treatment. A flower cluster is formed, and formation of the piece of sepal starts in a terminal flower bud (stage C). (**D**) 35 d after short day and high temperature (SDHT) 25/25 (SDHT condition, 8-h light at 25 °C/16-h dark at 25 °C) treatment. A flower cluster is developed (stage B2). (**E**) 30 d after SDHT 30/25 treatment, stage 0. (**F**) 30 d after short day and middle temperature (SDMT) 30/20 treatment, stage 0. (**G**) 30 d after SDLT 30/15 treatment, a flower cluster is developed (stage B2). (**H**) 30 d after short day and half low temperature 30/15/25 treatment. Formation of involucre becomes in a terminal flower bud (stage D). Stage 0; undifferentiated stage, stage A1–A3; initial to late differentiation stage, stage B1–B3; initial to late flower cluster formation stage, stage C; initial sepal formation stage, stage D; involucre formation stage. Scale bars = 1000 µm (**A** to **D**) or 500 µm (**E** to **H**).

Table 2. *FaTFL1–1* and *FaTFL2* expression determined by quantitative real-time polymerase chain reaction in crowns of *Fragaria ×ananassa* Tochiotome grown under different conditions for 0 to 35 d. The transcription relative to housekeeping gene *MSI1*. Means ± SE, *n* = 3.

Gene	Temperature	0 d[z]	20 d	25 d	30 d	35 d
FaTFL1–1	SDHT 30/25	1.00	0.45 ± 0.24	0.21 ± 0.07	0.27 ± 0.14	0.21 ± 0.03
	SDMT 30/20	1.00	0.30 ± 0.12	0.05 ± 0.02	0.02 ± 0.00	0.12 ± 0.02
	SDLT 30/15	1.00	0.25 ± 0.10	0.17 ± 0.05	0.30 ± 0.06	0.23 ± 0.14
	SDHLT 30/15/25	1.00	0.28 ± 0.08	0.34 ± 0.14	0.35 ± 0.14	0.15 ± 0.05
FaTFL2	SDHT 30/25	1.00	0.43 ± 0.15	0.42 ± 0.23	0.25 ± 0.08	0.33 ± 0.13
	SDMT 30/20	1.00	0.41 ± 0.09	0.24 ± 0.11	0.20 ± 0.12	0.28 ± 0.10
	SDLT 30/15	1.00	0.06 ± 0.01	0.07 ± 0.03	0.17 ± 0.09	0.10 ± 0.02
	SDHLT 30/15/25	1.00	0.07 ± 0.02	0.04 ± 0.01	0.04 ± 0.02	0.08 ± 0.05

[z]Average expression values of each gene in three biological replicates were calculated regardless of the treatments and were set as 1-fold. SDHT = short day and high temperature; SDMT = short day and middle temperature; SDLT = short day and low temperature; SDHLT = short day and half low temperature.

15 °C) in summer to promote floral bud initiation. Previously, we found that floral bud differentiation occurred under short day and high temperature conditions (SDHT 25/25; an 8-h light, 25 °C/ a 16-h dark 25 °C) (Fig. 2D; Nakajima et al., 2014). As the down regulation of *FaTFL2* gene responsible for floral bud initiation occurred dependently of temperature rather than photoperiod, we investigated the floral bud development under short day and different temperature conditions.

As shown in Table 2, the expression level of *FaTFL2* was markedly reduced after 20 and 25 d at SDLT 30/15 (an 8-h light, 30 °C/ a 16-h dark, 15 °C; used as a positive control) and SDHLT 30/15/25 (an 8-h light, 30 °C/an 8-h dark, 15 °C/an 8-h dark, 25 °C) compared with day 0. We observed flower buds at stages 0 (undifferentiated), A3 and C (differentiated) on samples after 25, 30, and 35 d of SDLT 31.2/15, respectively, in 2013 (Fig. 2A–C), and observed those of stages B2 and D (differentiated) on samples after 30 d of SDLT 30/15 and SDHLT 30/15/25, respectively, in 2014 (Fig. 2G and H). Contrarily, no flower buds were observed on samples after 30 d at SDHT 30/25 (an 8-h light, 30 °C/a 16-h dark, 25 °C; used as a negative control) and SDMT 30/20 (an 8-h light, 30 °C/a 16-h dark, 20 °C) in 2014 (Fig. 2E and F). The samples after 35 d of SDLT 30/25 also showed flower buds of undifferentiated stage 0, however, some samples after 35 d of SDMT 30/20 showed the 0 ≈ A1 stage (results not shown). Although it was difficult to judge exactly either the undifferentiated stage 0 or the incipient beginning of flower bud formation at stage A1, the floral bud differentiation might occur under the SDMT 30/20 condition. In case of SDHT 30/25 (an 8-h light, 30 °C/a 16-h dark, 25 °C), 57% and 58% reduction of the *FaTFL2* expression was observed at 20 and 25 d compared with day 0, which was lower compared with 93% and 94% reduction of SDLT 30/15 and SDHLT 30/15/25 (Table 2). The 57% and 58% reduction of *FaTFL2* expression seemed to be inadequate for flower bud initiation. The same reduction level (59%) of the *FaTFL2* expression after 20 d compared with day 0 was also observed in SDMT 30/20, however, greater reduction of 76% was observed after 25 d compared with day 0. Stages 0 ≈ A1 at 35 d in SDMT 30/20 might be caused by this reduction and/or the *FaTFL1–1* reduction (Table 2).

In our previous results, no reduction of *FaTFL1–1* at 20 to 35 d after the treatments was observed under the flower inductive SDLT 31.2/15 condition (Nakajima et al., 2014), suggesting that the gene was expressing mainly in the area of the crown of no correlation with flower bud differentiation. However, we observed the reduction of *FaTFL1–1* expression at 20 and 25 d of both the noninductive and inductive conditions, that is, 55% to 83% reduction, and the most severe reduction was observed at 25 to 35 d of SDMT 30/20 (Table 2). The difference of the expression level of *FaTFL1–1* might be from the difference of the maximum temperature condition between SDLT 31.2/15 and short day (SD) temperatures of 30/25, 30/20, 30/15, and 30/15/25. In the SDLT 31.2/15 condition, average temperature outside of 0900 HR to 1700 HR was 31.2 °C, which is close to the 30 °C of SD-temperatures of 30/25, 30/20, 30/15, and 30/15/25, but rising to 36.1 °C at 1400 HR at the end of July. We observed the reduction of *FaTFL1–1* expression at temperate conditions not rising more than 30 °C, and the severe reduction of *FaTFL1–1* in this condition (95% at 25 d) might relate to the stage 0 ≈ A1 at 30 d of SDMT 30/20 (Table 1).

Interestingly, although the average temperatures of SDMT 30/20 and SDHLT 30/15/25 are the same (23.3 °C), the marked reduction of the *FaTFL2* gene with clear flower bud differentiation occurred at SDHLT 30/15/25, not SDMT 30/20, suggesting that temperature drops below 15 °C at dark after 30 °C at light might be important for *FaTFL2* reduction and floral bud differentiation. Since flower buds were induced under the SDHLT 30/15/25 conditions in addition to the SDLT 30/15 conditions used as a positive control, half reduction of the energy cost would be possible if the temperature of 25 °C at the 8-h dark period could be maintained without cooling.

The plants are grown in the field from 0900 HR to 1700 HR with the temperature rising more than 30 °C (average 31.2 °C), then sheltered in a curtained tunnel tent with air cooling (15 °C) from 1700 HR to 0900 HR in the case of normal super-forcing cultivation. Since the average temperature outside at 0100 HR to 0900 HR is 26.7 °C which is close to 25 °C, it might be possible to turn off the switch for cooling in a curtained tunnel tent during 0100 HR to 0900 HR. This means half reduction of the energy costs of super-forcing strawberry cultivation. Moreover, more than 93% *FaTFL2* reduction at 20 and/or 25 d after treatment of SDHLT could provide an alternative method to a conventional microscopic observation of a growing point for determining whether floral differentiation occurred, since a number of samples can be analyzed at one time once some molecular biological tools are set up.

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