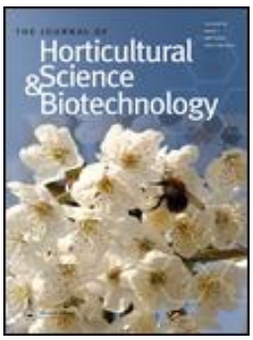

The Journal of Horticultural Science and Biotechnology

ISSN: 1462-0316 (Print) 2380-4084 (Online) Journal homepage: http://www.tandfonline.com/loi/thsb20

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To cite this article: Y. Nakano, Y. Higuchi, K. Sumitomo, A. Oda, T. Hisamatsu & Naro (2015) Delay of flowering by high temperature in chrysanthemum: heat-sensitive time-of-day and heat effects on CsFTL3 and CsAFT gene expression, The Journal of Horticultural Science and Biotechnology, 90:2, 143-149, DOI: 10.1080/14620316.2015.11513165

To link to this article: http://dx.doi.org/10.1080/14620316.2015.11513165

Published online: 07 Nov 2015.

Journal of Horticultural Science & Biotechnology (2015) **90** (2) 143–149

Delay of flowering by high temperature in chrysanthemum: heat-sensitive time-of-day and heat effects on *CsFTL3* and *CsAFT* gene expression

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(Accepted 7 November 2014)

SUMMARY
High temperature-induced delay of flowering is a major problem during the production of short-day (SD) plants such as *Chrysanthemum*. We have investigated the heat-sensitive hours for flowering in *C. seticuspe*. Heat treatment at 30ºC was applied for 16, 8, or 4 h d^{-1}, at different times of the day, under SD conditions (8 h photoperiod from 08.00 – 16.00 h), using 20ºC as the optimum temperature. Heating between midnight and dawn (00.00 – 08.00 h) delayed floral initiation for up to 2 d, but delayed flowering for > 25 d, which represented the heat-sensitive hours for capitulum development. Heating during this period suppressed expression of the *FLOWERING LOCUS T-like3* (*CsFTL3*) gene, a promoter of flowering induced in leaves by SDs. However, expression of *CsFTL3* was higher compared to non-floral-inductive photoperiods with a night-break. The *anti-florigenic FT/TFL1 family protein* gene *(CsAFT)* is an inhibitor of flowering induced in leaves by long-days. Expression of *CsAFT* remained low during heating under SDs, even when the plants flowered late. Our results show that high temperatures had little effect on floral initiation, which required suppression of *CsAFT* and a slight induction of *CsFTL3* gene expression under SDs. In contrast, a high temperature between 00.00 – 08.00 h appeared to delay flowering by decreasing the level of expression of *CsFTL3* required for capitulum development.

Flowering in *Chrysanthemum morifolium* Ramat. is primarily regulated by day-length. This qualitative short-day (SD) plant flowers naturally during the Autumn. Year-round flower production requires a night-break (NB), with artificial lighting, to inhibit flowering, and SD treatment to promote flowering. The shoot apical meristem (SAM) in chrysanthemum is converted into an inflorescence meristem after transition to the reproductive stage. Floral meristems appear on the growing inflorescence meristem, and develop into florets during capitulum development. Long-day (LD) or NB conditions inhibit both floral initiation and capitulum development (Adams *et al*., 1996). Continuous SD conditions are required for flowering. However, in practice, the growth temperature under SD conditions affects the accuracy of photoperiod-mediated scheduling of flowering. Because the optimum flowering temperature for chrysanthemum is approx. 20ºC (Whealy *et al*., 1987), the delay in flowering caused by supra-optimum temperatures (e.g., 30ºC) in Summer presents a major problem to growers and production at low- and mid-latitudes (Shibata, 1997; Nozaki and Fukai, 2008).

Previous studies have shown that the high temperature-induced delay in flowering is caused by inhibition of capitulum development, rather than by inhibition of floral initiation (Nozaki and Fukai, 2008; Nakano *et al*., 2013). It has also been reported that high night-temperatures delay flowering (Cockshull and Kofranek, 1994), while extending the dark period by shading with blackout curtains is commonly used as a SD treatment during LD seasons. This treatment may warm the glasshouse to unfavourably high night temperatures, because it reduces ventilation (Shibata, 1997). Compared to photoperiod control (i.e., lighting and shading), temperature control is expensive and energy-consuming. Thus, improving our understanding of how high temperatures affect flowering would help increase productivity and lead to more efficient energy use in chrysanthemum production systems.

Chailakhyan (1936) first suggested the existence of "florigen", a promoter of flowering that transmitted the photoperiodic flower-inducing stimulus perceived in the leaves to the SAM. Genes that encode phosphatidylethanolamine binding-like proteins (PEBP) have been identified as florigens. These include *FLOWERING LOCUS T (FT)* in *Arabidopsis thaliana* (Kardailsky *et al*., 1999; Kobayashi *et al*., 1999; Corbesier *et al*., 2007) and *Heading date 3a* (*Hd3a*) in *Oryza sativa* (Kojima *et al*., 2002; Tamaki *et al*., 2007). In chrysanthemum, an *FT-like3* gene encodes a flowering promoter induced by SDs in the leaves (*CmFTL3* in *C. morifolium* and CsFTL3 in *C. seticuspe*; Higuchi *et al*., 2012; Oda *et al*., 2012; Sumitomo *et al*., 2012). In LD and SD plants such as *A. thaliana* (King *et al*., 2008) and *Pharbitis nil* (Hayama *et al*., 2007), a single exposure to a floral-inducing photoperiod induces a high level of *FT* gene expression and flower development. However, *Cm*/*CsFTL3* was induced only slightly by a single SD treatment (Oda *et al*., 2012). In accordance with the requirement for continuous SD for flowering (Adams *et al*., 1996), *Chrysanthemum* spp. require repeated SDs to

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express *CmFTL3* or *CsFTL3* at high levels (Higuchi *et al*., 2013; Nakano *et al*., 2013). During high temperature-induced delay of flowering, up-regulation of *Cm*/*CsFTL3* in leaves is inhibited (Nakano *et al*., 2013). The amount of CmFTL3 or CsFTL3 protein synthesised at high temperatures appeared to be insufficient for downstream gene regulation (Nakano *et al*., 2013). Photoperiod regulates *Cm*/*CsFTL3* gene expression by measurements of the dark-time, starting at the "lights-off" signal (Higuchi *et al*., 2013). The effects of high temperature on *Cm*/*CsFTL3* gene expression should therefore be studied in relation to circadian periodicity.

TERMINAL FLOWER 1 (*TFL1*) encodes a PEBP-family protein in *A. thaliana* that is specifically expressed in the inflorescence meristem to inhibit determinate flowering by antagonising *FT* expression (Ratcliffe *et al*., 1998; Hanano and Goto, 2011). It is unlikely that the *TFL1* homologue in chrysanthemum is involved in the high temperature-induced delay of flowering, as its expression in the shoot tip is temperature-insensitive (Nakano *et al*., 2013). Recently, anti-florigen, a novel systemic floral repressor (anti-florigenic FT/TFL1 family protein; AFT), was identified in *C. seticuspe* (Higuchi *et al*., 2013). CsAFT also belongs to the PEBP-family. The *CsAFT* gene is transcribed mainly in leaves following LD or NB stimuli, with the resulting polypeptide being transported to the SAM (Higuchi *et al*., 2013). CsAFT appears to maintain vegetative growth of the SAM by antagonising any residual CsFTL3 protein under non-inductive photoperiods. Studies are therefore required to determine whether the high temperature-induced delay of flowering in *Chrysanthemum* spp. involves this type of photoperiodic maintenance system (i.e., vegetative *vs*. reproductive growth).

C. seticuspe is a diploid (2n = 18) chrysanthemum species that has been used as a model with which to study day-length and high-temperature responses using molecular techniques (Oda *et al*., 2012; Higuchi *et al*., 2013; Nakano *et al*., 2013). *C. seticuspe* tends to be used in preference to *C. morifolium*, which is a more complex hexaploid (2n = 54) species. In this study, the high temperature-sensitive time for flowering in *C. seticuspe* was studied using several different heat treatments. The effects of heat treatment on *CsFTL3* and *CsAFT* gene expression were also investigated.

MATERIALS AND METHODS

Plant material

C. seticuspe (Maxim.) Hand.-Mazz. f. boreale (Makino) H. Ohashi & Yoneke Accession NIFS-3 was used for these experiments. Mother stock plants and nursery plants were grown in a glasshouse that was heated below 18ºC and ventilated above 25ºC. In addition to natural light, a 4 h NB (23.00 – 03.00 h) was provided by fluorescent tubes (EFR25ED/22; Toshiba Lighting & Technology Corp., Kanagawa, Japan) to inhibit flowering. Cuttings (5 cm in length) were rooted in 1.5-cm diameter plastic-cell trays filled with Metro-Mix 350 (Sun-Gro Horticulture, Agawam, MA, USA). Rooted cuttings were cold-treated at 5ºC under a 16 h photoperiod for 4 – 5 weeks to avoid dormancy. The plants were then transplanted to 6-cm diameter plastic pots containing a garden soil (Yokabaido; Hokkaido Peatmoss Co. Ltd., Hokkaido, Japan), and were allowed to establish for 1 – 2 weeks.

Zeitgeber Time (ZT), which is a 24 h time-scale in which "light on" is ZT 0 in photoperiodic research, was employed after the potted plants were transferred to a growth chamber (LPH-350SP; NK Systems, Osaka, Japan). The plants were acclimatised at 20ºC for 1 week in the chamber, with 70% relative humidity, under an 8 h photoperiod (ZT 0 – 8) and a 4 h NB (ZT 14 – 22). Light was supplied by FHF32EX-N-HX-S fluorescent tubes (NEC Lighting Ltd., Tokyo, Japan) at a photosynthetic photon flux density (PPFD) of 200 μmol m^{-2} s^{-1}.

Temperature treatments

The high-temperature treatment was conducted in the growth chamber under an 8 h light period (ZT 0 – 8) and 16 h dark period (ZT 8 – 24), without a NB treatment, for 70 d. Control plants were grown continuously at 20ºC in all experiments.

In Experiment 1, 16 h of heat treatment at 30ºC was applied from lights-on to the first half of the dark period (i.e., ZT 0 – 16) or during the light period and the second half of the dark period (i.e., ZT 0 – 8 and 16 – 24). Plants were also grown at a constant 30ºC.

In Experiment 2, 8 h of 30ºC heat treatment was applied during the light period (ZT 0 – 8), the first (ZT 8 – 16), or the second half of the dark period (ZT 16 – 24).

In Experiment 3, 4 h of 30ºC heat treatment was applied after lights-off (i.e., ZT 8 – 12), at midnight (ZT 14 – 18), or before lights-on (ZT 20 – 24).

The dates when the involucre became visible and the first ligulate flower stood vertically were recorded as days to visible capitulum (DVC) and flowering (DF), respectively.

Total RNA preparation and reverse transcription-qPCR

Before the start of the high-temperature treatment (NB conditions, 20ºC, ZT 0) and after 14 d of each temperature treatment (at ZT 0, 4, 8, 12, 16, or 18), expanded leaves were collected from the same order of nodes, frozen in liquid nitrogen, and stored at –80ºC until use. Each leaf sample was ground to a fine powder in a Shake Master homogeniser (Bio Medical Science Inc., Tokyo, Japan). Total RNA was extracted from approx. 20 mg of each frozen powder using the RNeasy Plant Mini Kit (Qiagen K. K., Tokyo, Japan), in combination with RNase-free DNase (Qiagen K. K.), according to the manufacturer's instructions. The purity and concentration of each total RNA sample were measured using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific K. K., Kanagawa, Japan). The amount of residual genomic DNA was low enough not to affect mRNA detection by PCR.

Reverse transcription (RT) was performed using 250 ng of total RNA and PrimeScript RT Master Mix Perfect Real Time (TaKaRa-BIO Inc., Shiga, Japan), according to the manufacturer's instructions. The resulting cDNA was diluted to 10% (v/v) in 10 mM Tris-HCl buffer (pH 8.0), containing 1 mM EDTA.

Each quantitative-polymerase chain reaction (qPCR) was performed using SYBR Premix Ex Taq II Tli RNaseH plus (TaKaRa BIO Inc.) in a Thermal Cycler Dice Real Time System II (TaKaRa BIO Inc.). The qPCR conditions were: 1 min of denaturation at 95ºC,

TABLE I
Effect of daily heating treatment at 30ºC on flowering in Chrysanthemum seticuspe

Experiment	Heating period*	No. of nodes[#]	DVC[‡]	DF[‡]
Experiment 1	Control (20ºC constant)	15.1 ± 0.8 a[†]	19.9 ± 0.9 a	54.9 ± 1.3 a
(16 h heating)	ZT 0 – 16	15.9 ± 1.3 ab	20.7 ± 0.7 a	57.2 ± 1.7 b
	ZT 0 – 8 and 16 – 24	16.7 ± 1.0 b	19.5 ± 0.5 a	>71[¶] c
	30ºC constant	16.3 ± 0.9 b	20.0 ± 0.9 a	>71 c
Experiment 2	Control (20ºC constant)	13.4 ± 0.5 a	21.0 ± 1.0 a	54.6 ± 1.8 a
(8 h heating)	ZT 0 – 8	13.6 ± 0.8 a	20.9 ± 0.9 a	58.5 ± 1.6 b
	ZT 8 – 16	13.4 ± 0.5 a	20.5 ± 0.7 a	60.0 ± 2.4 b
	ZT 16 – 24	14.4 ± 0.6 b	23.0 ± 1.5 b	>71 c
Experiment 3	Control (20ºC constant)	14.0 ± 1.2 a	19.5 ± 1.3 a	48.3 ± 1.1 a
(4 h heating)	ZT 8 – 12	14.3 ± 0.9 a	19.3 ± 1.1 a	49.0 ± 1.0 a
	ZT 14 – 18	14.9 ± 0.6 a	20.5 ± 1.5 ab	61.2 ± 1.8 b
	ZT 20 – 24	14.7 ± 0.6 a	21.2 ± 1.2 b	65.8 ± 1.6 b

*Photoperiod was 8 h light (ZT 0 – 8) and 16 h dark (ZT 8 – 24).
[#]Number of nodes that developed until floral initiation. The uppermost visible node was marked and set to 0 at the start of each experiment.
[†]Mean values ± SD (n = 18 in Experiment 1; n = 16 in Experiment 2; and n = 12 in Experiment 3). Mean values followed by different lower-case letters in each column in the same Experiment differed significantly at $P \leq 0.05$ by the Steel-Dwass test.
[¶]None of the plants flowered within the 70 d experimental period.
[‡]DVC, days to visible capitulum; DF, days to flowering.

followed by 40 cycles at 95ºC for 5 s and at 60ºC for 30 s, then by melt curve analysis of the PCR product.

CsFTL3 (GenBank Accession No. AB679272), *CsAFT* (AB839766) and *CsACT* (AB770470) were amplified using primer pairs, the accuracy and efficiency of which had previously been verified (Higuchi *et al*., 2013; Nakano *et al*., 2013). The data were normalised based on the level of expression of the *actin* gene (*CsACT*). The Cycle Threshold (CT) of *CsACT* did not differ among the cDNA samples generated from 250 ng total RNA. ΔCT was obtained by subtracting CT_{CsACT} from CT_{CsFTL3} and CT_{CsAFT}. ΔCT values in leaves collected before the start of the heat treatment (NB conditions, 20ºC, ZT 0) were used as a reference, and set at 1.0. The ΔΔCT value for each gene was obtained by subtracting $\Delta CT_{reference}$ from ΔCT_{sample}. The data were expressed using $2^{-\Delta\Delta C_T}$ (Livak and Schmittgen, 2001).

Three biological replicates were used for cDNA preparation, with PCR being used to analyse two technical replicates of each cDNA. Each PCR product was checked by melt curve analysis.

RESULTS

Experiment 1: 16 h heating d^{-1}

Compared to the 20ºC controls, the 16 h heat-treatment from lights-on to midnight (ZT 0 – 16) slightly increased DF by approx. 2 d (Table I). *CsFTL3* gene expression in leaves after 14 SDs was slightly lower in these plants compared to control plants (Figure 1A). Compared to the controls, heat treatment during the light period and the second half of the dark period (i.e., ZT 0 – 8 and ZT 16 – 24) slightly increased the number of nodes (Table I). These plants did not flower during the experimental period. In this treatment, *CsFTL3* gene expression was reduced to its lowest level, but was still two-to-three-fold higher than in plants under NB conditions (Figure 1A). Compared to 20ºC control plants, the flowering nodes were slightly larger in plants maintained at a constant 30ºC, but these plants did not flower during the experimental period (Table I). Constant heating at 30ºC decreased *CsFTL3* gene expression (Figure 1A). Compared to NB conditions, *CsAFT* gene expression in leaves decreased under SDs, irrespective of heat treatment (Figure 1B).

Experiment 2: 8 h heating d^{-1}

Compared to the 20ºC controls, 8 h heat treatment at 30ºC during the light period (ZT 0 – 8) and the first half of the dark period (ZT 8 – 16) increased DF by a few days (Table I). After 14 SDs, *CsFTL3* gene expression in the leaves of these plants was lower than in the controls

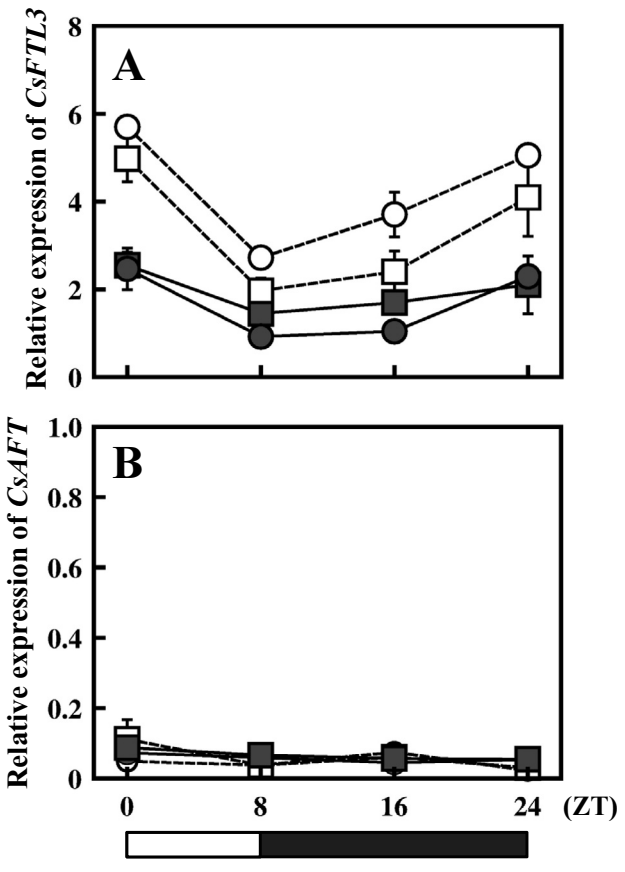

FIG. 1
Effects of daily 16 h heating at 30ºC on *CsFTL3* (Panel A) and *CsAFT* (Panel B) gene expression after 14 short-days (SDs). The photoperiod was 8 h light (ZT 0 – 8) and 16 h dark (ZT 8 – 24). Open circles, control plants at a constant 20ºC; open squares, plants heated from ZT 0 – 16; solid circles, plants heated from ZT 0 – 8 and ZT 16 – 24; solid squares, plants at a constant 30ºC. The level of gene expression in leaves collected before the start of each heat treatment (i.e., NB conditions, 20ºC, ZT 0) was set at 1.0 by the ΔΔCT method. Values are means ± SE (n = 3). Open and closed bars at the bottom indicate light and dark periods, respectively.

during the day and the night; however, the levels were similar at "lights-on" (Figure 2A). Plants heat-treated during the second half of the dark period (ZT 16 – 24) resulted in a larger number of nodes and DVC (Table I). These plants did not flower during the experimental period. *CsFTL3* gene expression in these plants was significantly lower than in the other treatments, but was slightly higher than that in plants under NB conditions (Figure 2A). Lower *CsAFT* gene expression in leaves was recorded for all temperature conditions tested compared to NB conditions (Figure 2B).

Experiment 3: 4 h heating d^{-1}

DF values in plants that were heat-treated after "lights-off" (ZT 8 – 12) did not differ from those in control plants (Table I). After 14 SDs, *CsFTL3* gene expression in the leaves of these plants was similar to that in control plants (Figure 3B). DF values in the 4-h heat treatment increased by 13 d when heat was applied in the middle of the night (ZT 14 – 18) and by 17 d when heat was applied before lights-on (ZT 20 – 24; Table I). The 4 h heat treatment before "lights-on" increased DVC values slightly. *CsFTL3* gene expression in these plants was lower than in control plants, but was higher than in plants under NB conditions (Figure 3A). Compared to NB plants, *CsAFT* gene expression was significantly reduced in leaves under SDs, irrespective of the temperature treatment (Figure 3B).

DISCUSSION

Understanding how high temperature affects flowering is important in order to establish an accurate system to regulate flowering in chrysanthemum. In this study, the second-half of the night was identified as the heat-sensitive hours for flowering in *C. seticuspe*. Here, we discuss the mechanisms of this high temperature-induced flowering delay in the context of expression of the genes, *CsFTL3* and *CsAFT*.

Experiments were conducted under controlled environment conditions, and the results showed that a high temperature between midnight and dawn severely delayed flowering in *C. seticuspe* (Table I). Four hours of heating around midnight had almost the same inhibitory effect on flowering as 4 h heating before dawn, although 8 h or 4 h of heating after dusk affected DF values to a lesser extent (Table I). These results indicate that the high temperature-sensitive time of day that critically delayed flowering started at around midnight. The products of the *Cm*/*CsFTL3* gene(s) acted as SD-

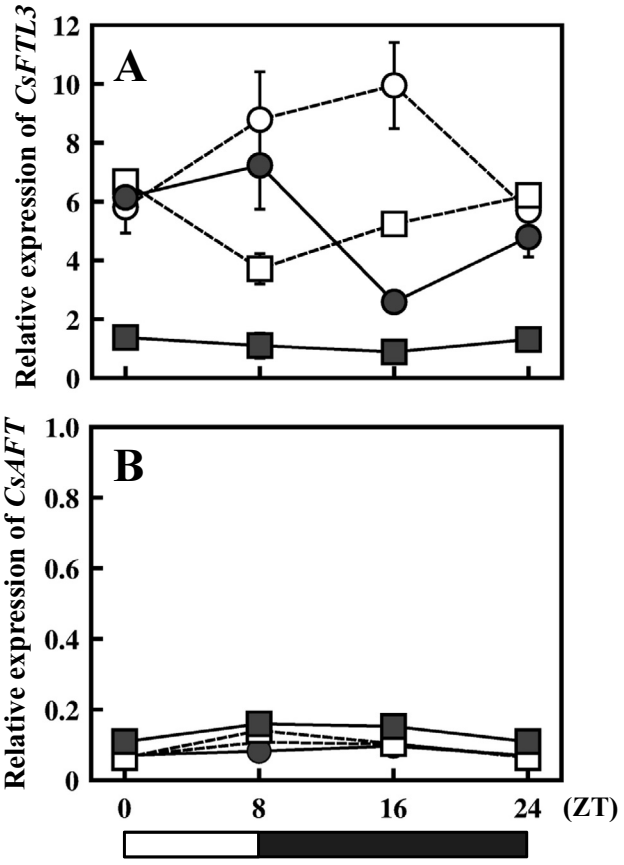

FIG. 2
Effects of daily 8 h heating at 30ºC on *CsFTL3* (Panel A) and *CsAFT* (Panel B) gene expression after 14 short-days (SDs). The photoperiod was 8 h light (ZT 0 – 8) and 16 h dark (ZT 8 – 24). Open circles, control plants at a constant 20ºC; open squares, plants heated from ZT 0 – 8; solid circles, plants heated from ZT 8 – 16; solid squares, plants heated from ZT 16 – 24. The level of gene expression in leaves collected before the start of each heat treatment (i.e., NB conditions, 20ºC, ZT 0) was set at 1.0 by the ΔΔCT method. Values are means ± SE (n = 3). Open and closed bars at the bottom indicate light and dark periods, respectively.

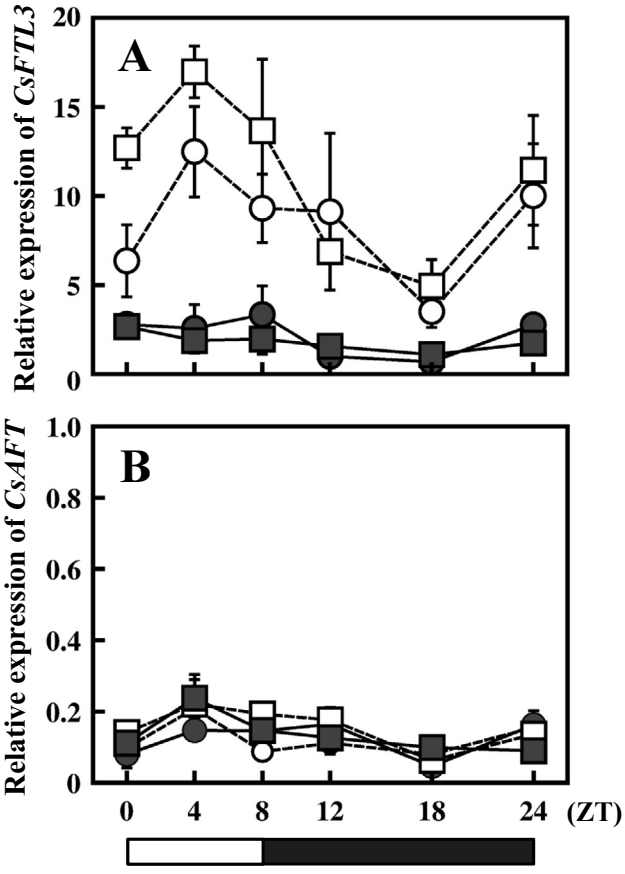

FIG. 3
Effects of daily 4 h heating at 30ºC on *CsFTL3* (Panel A) and *CsAFT* (Panel B) gene expression after 14 short-days (SDs). The photoperiod was 8 h light (ZT 0 – 8) and 16 h dark (ZT 8 – 24). Open circles, control plants at a constant 20ºC; open squares, plants heated from ZT 8 – 12; solid circles, plants heated from ZT 14 – 18; solid squares, plants heated from ZT 20 – 24. The level of gene expression in leaves collected before the start of each heat treatment (i.e., NB conditions, 20ºC, ZT 0) was set at 1.0 by the ΔΔCT method. Values are means ± SE (n = 3). Open and closed bars at the bottom indicate light and dark periods, respectively.

induced floral promoters in chrysanthemum, the expression of which remained low under non-inductive photoperiodic conditions (Higuchi *et al*., 2012; Oda *et al*., 2012; Sumitomo *et al*., 2012). High temperature delayed flowering by reducing the expression of *Cm*/*CsFTL3*, which was alleviated by high *CmFTL3* gene expression in *C. morifolium*. This phenomenon illustrated the quantitative correlation between flowering and the level of *Cm*/*CsFTL3* gene expression (Nakano *et al*., 2013). Thus, the lower the level of *CsFTL3* expression (Figure 1A; Figure 2A; Figure 3A), the greater the increase in DF values (Table I). The results also indicated that heat-sensitive mechanism(s) are involved in the increase in *CsFTL3* gene expression between midnight and dawn (ZT 16 – 24)

High temperature delayed flowering by inhibiting capitulum development, rather than by inhibiting floral initiation in chrysanthemum (Nozaki and Fukai, 2008; Nakano *et al*., 2013). In this study, the number of nodes was not affected, or increased only slightly by heat treatment (Table I). It has also been reported that although the number of nodes increased under high temperature conditions in *C. seticuspe*, high temperature had little effect on the timing of floral initiation, because the plastochron was shortened under warmer conditions (Nakano *et al*., 2013). Thus, the timing of floral initiation was minimally affected by the heat treatments, some of which delayed capitulum development considerably. High temperature is believed to delay capitulum development, and consequently flowering, by reducing expression of *Cm*/*CsFTL3* (Nakano *et al*., 2013). However, the recent discovery of *CsAFT*, an obligate floral inhibitor that supports the qualitative SD flowering response, prompted us to consider the role of this anti-florigen gene (Higuchi *et al*., 2013).

The *CsAFT* gene was expressed predominantly in leaves under non-inductive photoperiods to prevent the SAM from flowering by antagonising *CsFTL3* (Higuchi *et al*., 2013). *CsAFT* gene expression decreased independently of the duration or timing of high temperature under SDs (Figure 1B; Figure 2B; Figure 3B). Expression of *CsTFL1* (a functional orthologue of *TFL1* in *A. thaliana*) in shoot tips was temperature insensitive (Higuchi *et al*., 2013; Nakano *et al*., 2013). These results suggest that the high temperature-induced delay in flowering did not involve the activation of *CsAFT* in leaves, or *CsTFL1* in the SAM. Thus, the marked reduction in *CsAFT* expression by SD may lift the repression of floral initiation independently of temperature. Expression of *CsFTL3* in all heat-treated plants under SDs was slightly higher than under NB conditions (Figure 1A; Figure 2A; Figure 3A). In the constant heating experiment, expression of *CsFTL3* in plants heat-treated at 30ºC was similar to the level of expression in plants maintained at optimum temperatures over seven SDs, in which a terminal inflorescence meristem had already been observed (Nakano *et al*., 2013). The extent of *CsFTL3* expression induced by a few SDs, or expressed at basal levels, appeared to be sufficient to convert the SAM into inflorescence meristems in the absence of *CsAFT* expression. However, *CsFTL3* expression after floral initiation appeared to be insufficient to promote capitulum development in plants heat-treated between midnight and dawn (ZT 16 – 24).

After transferring the chrysanthemum plants from LD to SD conditions, the diurnal rhythm of *CsFTL3* gene expression peaked at around dawn (Oda *et al*., 2012). However, the diurnal peak was lost, or shifted, with an increase in *CsFTL3* gene expression under repeated SD cycles (e.g., see expression in control plants in Figure 1B, Figure 2B and Figure 3B; Higuchi *et al*., 2013; Nakano *et al*., 2013). This gradual increase and loss of diurnal rhythm in *Cm*/*CsFTL3* gene expression may be accounted for by both factors involved in gene regulation: (1) the photoperiod reception and time measurement systems in the SD pathway that lead to rhythmic *Cm*/*CsFTL3* expression; and (2) the late expression-enhancement mechanism that promotes *Cm*/*CsFTL3* expression, which depends on continuous SD signals. In other SD plants, *FT-like* gene expression clearly peaked around dawn under SD conditions, by a mechanism that sensed long periods of darkness and involved the circadian clock (e.g., in *O. sativa*, see Kojima *et al*., 2003; Ishikawa *et al*., 2011; in *P. nil*, see Hayama *et al*., 2007). In *C. seticuspe*, expression of *CsFTL3* under atypical conditions (16/14 L/D) was similar to that under a 24-h SD (10/14 L/D) photoperiod (Higuchi *et al*., 2013), indicating that day-length perception for *CsFTL3* regulation in chrysanthemum relied on the absolute duration of darkness, rather than on the length of the light period.

In this study, we observed that *CsFTL3* gene expression in plants under SDs was higher than in plants treated with a NB, irrespective of temperature (Figure 1A; Figure 2A; Figure 3A; Nakano *et al*., 2013), indicating that high temperature had only a slight effect on the SD-sensing required for the initial up-regulation of *CsFTL3* gene expression. Induction of *CsAFT* was triggered by light signals coinciding with the photosensitive phase set by the dusk signal, indicating that the time-keeping components set by the dusk signal may be involved in dark-time measurement (Higuchi *et al*., 2013). The temperature-independent repression of *CsAFT* expression under SDs (Figure 1B; Figure 2B; Figure 3B) also supported the theory that SD-sensing was not affected by high temperature. Compensating mechanisms for temperature changes in circadian clocks have been found in *A. thaliana* (Gould *et al*., 2006), which support this hypothesis. However, high temperature inhibited the amplification of *CsFTL3* under repeated SDs (Nakano *et al*., 2013). Plants heated between midnight and dawn expressed lower levels of *CsFTL3* throughout the day than those maintained at the optimum temperature (Figure 1A; Figure 2A; Figure 3A). Figure 2A shows that heating during the daytime, or during the first-half of the night, temporarily lowered *CsFTL3* gene expression in leaves collected soon after heating. These results indicate that the interaction between the SD pathway and the expression-enhancement mechanism of *CsFTL3* gene expression was sensitive to high temperature, and occurred mainly between midnight and dawn. To amplify *CsFTL3* gene expression under repeated SD cycles, we propose that a positive feedback loop exists, regulated by the CsFTL3/CsFDL1 complex, or by downstream target genes (Higuchi *et al*., 2013). Thus, the optimum temperature and continuous SD stimuli may be required for favourable feedback regulation that controls the

gradual amplification of *CsFTL3* gene expression, and contributes to capitulum development. The underlying mechanisms that govern *CsFTL3* gene expression require further study.

Heat-tolerant chrysanthemum cultivars have been bred commercially in Japan (Shibata, 1997). As indicated by Shibata (1997), the introduction of heat-tolerance into many cultivars, with other beneficial traits, has been indispensable for Summer production in warmer regions. One factor that contributed to heat tolerance was high levels of *CmFTL3* gene expression at high temperatures (Nakano *et al*., 2013). However, where the daily temperature always exceeded 25ºC, and rose to approx. 35ºC in Summer (for example, in Japan), even heat-tolerant cultivars exhibited delayed flowering. Thus, the combined use of heat-tolerant cultivars and more efficient environmental control could schedule more accurate flowering.

In Experiment 1, DF values increased only slightly with 16 h heating from dawn compared to the controls (Table I). Thus, the effect of high temperature was mitigated by 8 h of cooling from 30ºC to 20ºC from midnight to dawn. However, 8 h of cooling from dusk to midnight resulted in later flowering. SD treatments are regularly implemented by covering the glasshouse with black-out curtains to promote flowering under non-inductive photoperiods. However, this technique reduced ventilation and warmed the glasshouse (Shibata, 1997). To avoid delayed flowering, our results indicate that such a SD treatment should not be applied between midnight and dawn. Short duration thermal control, which may have similar effects to permanent thermal control, has been tested to save energy in glasshouse horticulture. The results obtained in the current study suggest that a brief cooling before dawn may alleviate the effects of high temperature during Summer.

CONCLUSION

This study showed that high temperatures from midnight to dawn (ZT 16 – 24) delayed flowering in *C. seticuspe*. The initial regulation of a floral promoter gene (*CsFTL3*) and a floral repressor gene (*CsAFT*) by SD was temperature-independent, triggering floral initiation, even when plants were grown at high temperatures. However, capitulum development (a later flowering event) was delayed by high temperatures from midnight to dawn, which inhibited the continued up-regulation of *CsFTL3*. These findings are expected to improve the efficiency of thermal control in glasshouses during chrysanthemum production.

This study was supported by a Grant-in-Aid from the Ministry of Agriculture, Forestry and Fisheries of Japan (Development of Mitigation and Adaptation Techniques to Global Warming in the Sectors of Agriculture, Forestry, and Fisheries). We thank S. Kamei and T. Hashimoto for their technical assistance.

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