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RESEARCH PAPER

CsFTL3, a chrysanthemum *FLOWERING LOCUS T-like* gene, is a key regulator of photoperiodic flowering in chrysanthemums

Atsushi Oda[1], Takako Narumi[2], Tuoping Li[1,*], Takumi Kando[1,†], Yohei Higuchi[1], Katsuhiko Sumitomo[1], Seiichi Fukai[2] and Tamotsu Hisamatsu[1,‡]

[1] National Institute of Floricultural Science (NIFS), National Agriculture and Food Research Organization (NARO), 2-1 Fujimoto, Tsukuba, Ibaraki 305-8519, Japan
[2] Graduate School of Agriculture, Kagawa University, 2393 Ikenobe, Miki-cho, Kita-gun, Kagawa 761-0795, Japan

* Present address: Liaoning University, Shenyang, Liaoning 110036, China
† Present address: Shimane Agricultural Technology Center, 2440 Ashiwata, Izumo, Shimane 693-0035, Japan
‡ To whom correspondence should be addressed. E-mail: tamotsu@affrc.go.jp

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Abstract

Chrysanthemum is a typical short-day (SD) plant that responds to shortening daylength during the transition from the vegetative to the reproductive phase. *FLOWERING LOCUS T* (*FT*)/*Heading date 3a* (*Hd3a*) plays a pivotal role in the induction of phase transition and is proposed to encode a florigen. Three *FT-like* genes were isolated from *Chrysanthemum seticuspe* (Maxim.) Hand.-Mazz. f. boreale (Makino) H. Ohashi & Yonek, a wild diploid chrysanthemum: *CsFTL1*, *CsFTL2*, and *CsFTL3*. The organ-specific expression patterns of the three genes were similar: they were all expressed mainly in the leaves. However, their response to daylength differed in that under SD (floral-inductive) conditions, the expression of *CsFTL1* and *CsFTL2* was down-regulated, whereas that of *CsFTL3* was up-regulated. *CsFTL3* had the potential to induce early flowering since its overexpression in chrysanthemum could induce flowering under non-inductive conditions. *CsFTL3*-dependent graft-transmissible signals partially substituted for SD stimuli in chrysanthemum. The *CsFTL3* expression levels in the two *C. seticuspe* accessions that differed in their critical daylengths for flowering closely coincided with the flowering response. The *CsFTL3* expression levels in the leaves were higher under floral-inductive photoperiods than under non-inductive conditions in both the accessions, with the induction of floral integrator and/or floral meristem identity genes occurring in the shoot apexes. Taken together, these results indicate that the gene product of *CsFTL3* is a key regulator of photoperiodic flowering in chrysanthemums.

Key words: *Chrysanthemum*, floral transition, flowering, *FT*, short day.

Introduction

Daylength plays an important role in floral transitions such as the shift from vegetative to inflorescence meristem identity in plants. In their pioneering work, Garner and Allard (1920) classified plants according to their responses to daylength. They showed that long-day (LD) plants take less time to flower when light exposure exceeds a certain critical daylength, while short-day (SD) plants flower earlier when light exposure is shorter than a certain critical daylength. The main site for the perception of daylength is recognized to be the leaf. Through tests with chrysanthemums,

Abbreviations: ABA, abscisic acid; bZIP, basic-leucine zipper; CaMV, *Cauliflower mosaic virus*; FAA, formalin–acetic acid–alcohol; GA, gibberellin; GUS, β-glucuronidase; LD, long day; LED, light-emitting diode; NB, night break; ORF, open reading frame; PEBP, phosphatidylethanolamine-binding protein; PPFD, photosynthetic photon flux density; QRT-PCR, quantitative real-time PCR; RACE, rapid amplification of cDNA ends; SAM, shoot apical meristem; SD, short day; SEM, scanning electron microscopy; ZT, Zeitgeber time.

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in 1936 Chailakhyan determined the leaf to be the site where a plant hormone called florigen is produced (for a review, see Chailakhyan and Krikorian, 1975). After a florigen is produced in the leaf, it travels through the phloem and into the shoot apical meristem (SAM), which is where flowering is initiated. This approach allows the process to be subdivided into two successive steps: (i) 'induction' mechanisms, which occur in the leaf, and (ii) floral 'evocation', which consists of the events occurring in the SAM that commit it to producing flowers (Evans, 1969).

Recent advances in molecular biology and traditional grafting studies have revealed that the gene product of *FLOWERING LOCUS T* (*FT*) in *Arabidopsis*, an LD plant, and that of *Heading date 3a* (*Hd3a*) in rice (*Oryza sativa* L.), an SD plant, can function as florigens to regulate flowering (Corbesier *et al.*, 2007; Mathieu *et al.*, 2007; Tamaki *et al.*, 2007; Notaguchi *et al.*, 2008). Light plays important roles in the regulation of flowering in *Arabidopsis* by regulating *CONSTANS* (*CO*) and *FT* (Kobayashi and Weigel, 2007). The expression of *CO* mRNA is under the control of the circadian clock. Post-translational regulation of the stability of the CO protein by light plays an important role in the regulation of *FT* expression (Suarez-Lopez *et al.*, 2001; Valverde *et al.*, 2004). Induction of flowering by FT-like proteins seems to be broadly conserved across plants. In tomato, a photoperiod-insensitive plant, *SINGLE FLOWER TRUSS* (*SFT*/*SP3D*), an orthologue of *FT*, is induced in mature leaves and triggers flowering (Lifschitz *et al.*, 2006). Grafting of shoots from *SFT*-overexpressing tomato plants to *sft* mutants rescued the late-flowering phenotype of the mutants, indicating that *SFT*-dependent graft-transmissible signals substituted for the developmental defects in the mutants; in addition, these signals substituted for LD stimuli in *Arabidopsis* and SD stimuli in Maryland Mammoth tobacco (Lifschitz *et al.*, 2006). This indicates that a systemic graft-transmissible floral signal is transmitted from the donor to the receptor and broadly conserved across plants (Shalit *et al.*, 2009). The fact that homologues of the FT protein are normally present in the phloem sap of cucurbits (Lin *et al.*, 2007) and rice (Aki *et al.*, 2008) supports the idea that FT-like proteins are universal signalling molecules travelling from the leaves to the meristems via phloem.

FT/Hd3a belongs to a small protein family whose members show homology to the mammalian phosphatidylethanolamine-binding protein (PEBP; Kardailsky *et al.*, 1999; Kobayashi *et al.*, 1999; Kojima *et al.*, 2002). In *Arabidopsis*, in addition to *FT*, five PEBP family genes are found: *TERMINAL FLOWER 1* (*TFL1*), *TWIN SISTER OF FT* (*TSF*), *BROTHER OF FT AND TFL1* (*BFT*), *Arabidopsis thaliana CENTRORADIALIS homologue* (*ATC*), and *MOTHER OF FT AND TFL1* (*MFT*) (Bradley *et al.*, 1997; Mimida *et al.*, 2001). Phylogenetic analysis has resolved three major clades within this family, corresponding to *FT-like*, *TFL1-like*, and *MFT-like* genes. *FT-like* and *TFL1-like* genes function in controlling flowering time (Bradley *et al.*, 1997). *FT-like* genes promote flowering, whereas *TFL1-like* genes delay flowering and prevent conversion of the SAM into a floral meristem. In *Arabidopsis*, FT and TFL1 have antagonistic effects on flowering time, and their functions have been related to the presence of critical amino acid residues—Tyr85/Gln140 in FT and His88/Asp144 in TFL1 (Hanzawa *et al.*, 2005; Ahn *et al.*, 2006). In *Arabidopsis*, *FT* and its closest paralogue, *TSF*, play a similar role in inducing flowering and show similar patterns of regulation of responses to photoperiod and vernalization (Yamaguchi *et al.*, 2005). Rice is classified as a facultative SD plant; its flowering is not completely suppressed under LD conditions. It uses two florigen genes, *Hd3a* and its closest paralogue, *Rice flowering locus T1* (*RFT1*), depending on daylength (Komiya *et al.*, 2008, 2009). *Hd3a* is the major floral activator under SD conditions, while *RFT1* is the major floral activator under LD conditions. Recently, a negative effect of *FT-like* genes on flowering has been postulated in sunflower and sugar beet. In sunflower, the in-frame allele of *Helianthus annuus FT1* (*HaFT1*) accelerates flowering and the frameshift allele represses flowering by interfering with the function of an activating paralogue, *HaFT4* (Blackman *et al.*, 2010). In sugar beet, *Beta vulgaris FT2* (*BvFT2*) accelerates flowering, and its paralogue, *BvFT1*, represses flowering. A comparison analysis of BvFT1 and BvFT2 revealed that substitutions in the fourth exon, encoding an external loop of PEBP, are essential for the antagonistic functions (Pin *et al.*, 2010). *MFT-like* genes may play different roles in plant development, compared with *FT-like* and *TFL1-like* genes. MFT regulates seed germination via the modulation of abscisic acid (ABA) and gibberellin (GA) signalling in *Arabidopsis* (Xi *et al.*, 2010).

FT is transported from phloem companion cells to shoot apexes via the phloem and interacts with a basic-leucine zipper (bZIP) transcription factor, FLOWERING LOCUS D (FD) (Abe *et al.*, 2005; Wigge *et al.*, 2005; Corbesier *et al.*, 2007; Mathieu *et al.*, 2007; Tamaki *et al.*, 2007). Unlike FT, FD is only expressed at the shoot apexes and is independent of photoperiodic induction (Abe *et al.*, 2005; Wigge *et al.*, 2005). At the shoot apexes in *Arabidopsis*, FT and FD probably activate important regulators of floral fate, such as *APETALA1* (*AP1*) and *FRUITFULL* (*FUL*) (Kobayashi and Weigel, 2007). *FLORICAULA* (*FLO*) in snapdragon and its *Arabidopsis* orthologue, *LEAFY* (*LFY*), appear to play pivotal roles in specifying floral meristem identity (Weigel and Nilsson, 1995). *FLO*/*LFY* plays an important role in reproductive transition and regulates flower development by establishing the expression of floral organ identity genes (Benlloch *et al.*, 2007). The MADS-box genes *CAULIFLOWER* (*CAL*), *AP1*, and *FUL* act in a redundant manner to control meristem identity in *Arabidopsis*. *FUL* is expressed in inflorescence meristems and leaves, while *AP1* and *CAL* are preferentially expressed in inflorescences and floral meristems (Mandel *et al.*, 1992; Kempin *et al.*, 1995; Mandel and Yanofsky, 1995; Ferrándiz *et al.*, 2000). In addition to promoting floral identity, *FUL* also promotes floral transition (Ferrándiz *et al.*, 2000). From chrysanthemum, *CmSOC1*, *CmAFL1*, *CmFL*, and *CDM111* have been isolated as homologues of *SUPPRESSOR OF OVEREXPRESSION OF CO1* (*SOC1*), *AP1*/*FUL*, *FLO*/*LFY*, and *AP1*, respectively

(Shchennikova *et al.*, 2004; Li *et al.*, 2009). The expression of *CmSOC1*, *CmAFL1*, *CmFL*, and *CDM111* is induced in differentiating floral shoot apexes, suggesting that preserved mechanisms trigger reproductive transition in chrysanthemums (Shchennikova *et al.*, 2004; Li *et al.*, 2009; Sumitomo *et al.*, 2009).

Chrysanthemum (*Chrysanthemum morifolium* Ramat.) is a typical SD plant widely cultivated worldwide; variations in the flowering time of chrysanthemums (from early summer to winter) under natural conditions mainly reflect differences in their critical daylengths for flowering (Langton, 1977; Kawata, 1987). Seasonal changes in the extension growth and flowering of chrysanthemums are an adaptation to temperate climates at middle latitudes. Growth and flowering depend on the combination of growth temperature, daylength, and the environment in the previous season (for a review, see Cathey, 1969). Researchers have been trying to reveal how environmental conditions such as daylength and temperature regulate flowering, in order to achieve stable year-round flower production (Link, 1936; Post and Kamemoto, 1950; for a review, see Cathey, 1969). Chrysanthemum growers are currently trying to produce constantly throughout the year. However, in the case of year-round production, unexpected developments such as premature flower budding occasionally occur due to deviations from normal environmental conditions in greenhouses. Despite the efforts of researchers to understand the mechanisms underlying flowering in chrysanthemums, very little is known about floral transition and further development of floral meristems at the molecular level. *Chrysanthemum morifolium* is a complex hybrid derived from several species that grow in the wild in China and Japan (for a review, see Cathey, 1969), which makes its genetic analysis difficult. A wild diploid chrysanthemum, *Chrysanthemum seticuspe* (Maxim.) Hand.-Mazz. f. *boreale* (Makino) H. Ohashi & Yonek (*C. seticuspe* hereafter; $2n=18$), probably exhibits similar seasonal growth and flowering responses to other chrysanthemum cultivars. To overcome the issue of complex hybridity and polyploidy, *C. seticuspe* was used as an alternative model of chrysanthemum cultivars in this study.

The present study reports the isolation and functional analysis of homologues of *FT*/*Hd3a* from *C. seticuspe*. Ectopic expression of the *FT-like* gene *CsFTL3* promoted flowering in chrysanthemum under non-inductive conditions. The *CsFTL3* expression levels in the leaves of the two *C. seticuspe* accessions that differed in their critical daylengths for flowering closely coincided with the induction of the floral identity genes at the shoot apexes and capitulum development.

Materials and methods

Plant material and growth conditions

Chrysanthemum seticuspe accessions NIFS-3 and Matsukawa were used for the experiments. Stock plants were grown in a greenhouse maintained at an air temperature >18 °C and ventilated when the temperature increased above 25 °C, under a natural photoperiod with a 4 h night break (NB; 23:00–03:00 h) provided by incandescent lamps (K-RD100V60W; Matsushita Electric Industrial Co. Ltd, Osaka, Japan). Rooted cuttings from the stock plants were planted into 7.5 cm plastic pots containing a commercial horticultural soil (Kureha-Engei-Baido; Kureha Chemical Industry Co. Ltd, Tochigi, Japan) and grown in the greenhouse. When the plants developed four or five expanded leaves, they were transferred to a growth chamber maintained at 20 °C with a 16 h photoperiod (LD conditions). Light was supplied with fluorescent tubes (FL40SW; Mitsubishi Co. Ltd, Tokyo, Japan) at a photosynthetic photon flux density (PPFD) of 200 μmol m^{-2} s^{-1}. After 7 d of growth under these conditions, the plants were transferred to a growth chamber maintained at 20 °C with an 8 h photoperiod (SD conditions), a 16 h photoperiod (LD conditions), or an 8 h SD+an NB (NB conditions). Depending on the experiments, two kinds of NBs were given: a 4 h NB was supplied by fluorescent tubes at 200 μmol m^{-2} s^{-1} and a 15 min NB was supplied by red light-emitting diodes (LEDs) (LED-R; 660 nm; EYELA Co. Ltd, Tokyo, Japan) at 30 μmol m^{-2} s^{-1}.

CsFTL1 *cloning*

Total RNA was extracted from young fully expanded leaves and shoot apexes of *C. seticuspe* NIFS-3 by using the RNeasy Plant Mini Kit (QIAGEN K.K., Tokyo, Japan) and treated with RNase-free DNase (QIAGEN K.K.) according to the manufacturer's instructions. cDNAs were synthesized from 500 ng of total RNA by using the TaKaRa RNA PCR Kit (AMV) version 2.1 (TaKaRa BIO Inc., Shiga, Japan) according to the manufacturer's instructions; these cDNAs were used as templates in subsequent PCR experiments. PCR amplification was performed with an oligo(dT)-M13M4 adaptor primer (5′-GTTTTCCCAGTCACGAC-3′) and the degenerate primers (*FT*-F2: 5′-TAYACIYTIGTIATGGTI-GAYCC-3′; *FT*-R2: 5′-CCISWYTCICKYTGRCARTT-3′). PCR products ~320 bp long were cloned into the pGEM-T Easy Vector (Promega, Madison, WI, USA) as *CsFTL* fragments and sequenced. Internal gene-specific primers were designed to isolate full-length cDNAs of *CsFTL1*. The open reading frames (ORFs) of the genes were determined by 3′- and 5′-rapid amplification of cDNA ends-PCR (RACE-PCR), using the TaKaRa RNA PCR Kit (AMV) version 2.1 (TaKaRa BIO Inc.) and the Roche 5′/3′-RACE second-generation Kit (Roche Diagnostics K.K., Tokyo, Japan) according to the manufacturers' instructions. The full-length cDNAs of *CsFTL1* were amplified using the cDNA synthesized from the total RNA extracted from young fully expanded leaves as the template and cloned into the pGEM-T Easy Vector (Promega).

CsFTL2 *and* CsFTL3 *cloning*

Genomic DNA was extracted from the leaves of *C. seticuspe* NIFS-3 by using the DNeasy Plant Mini Kit (QIAGEN K.K.) according to the manufacturer's instructions. For *CsFTL2*, PCR amplification was performed with *CsFT*-F (5′-ATGCCG AGGGAAAGGGATCC-3′) and *CsFT*-1R (5′-AGCTCCTG-TAGTCTCTGGAA-3′) primers based on the *CsFTL1* cDNA sequence. PCR products ~1500 bp long were cloned into the pGEM-T Easy Vector (Promega) and sequenced. Clones that were even slightly different from *CsFTL1* were designated *CsFTL2*.

For *CsFTL3*, PCR amplification was performed with *CsFT*-F (5′-ATGCCGAGGGAAAGGGATCC-3′) and *CsFT*-2R (5′-CCCAATTGCCGGAATAGCAC-3′) primers. PCR products ~380 bp long were cloned into the pGEM-T Easy Vector (Promega) and sequenced. Clones that were even slightly different from both *CsFTL1* and *CsFTL2* were designated *CsFTL3*.

For full-length cDNAs of *CsFTL2* and *CsFTL3*, internal gene-specific primers, *CsFTL2*-F (5′-TGGGTGCGATCT-CAAACCCTCTCAGA-3′) and *CsFTL3*-F (5′-ACTTACACTG-GTTGGTTACC-3′), were used for 3′-RACE-PCR. The products were cloned into the pGEM-T Easy Vector (Promega) and sequenced.

Comparison of CsFTL genes and phylogenetic analysis

The sequences of the *CsFTL* genes were aligned with ClustalX, and K_s values were calculated with Molecular Evolutionary Genetics Analysis version 5 (MEGA 5) (http://www.megasoftware.net). The amino acid sequences of the PEBP family were assembled with ClustalX. A Neighbor–Joining phylogenetic tree was generated with MEGA 5, using the Poisson model with gamma-distributed rates and 1000 bootstrap replicates.

Expression analysis by quantitative real-time PCR

The abundance of transcripts was investigated by quantitative real-time PCR (QRT-PCR). Total RNA was extracted from each tissue by using the RNeasy Plant Mini Kit (QIAGEN K.K.) and treated with RNase-free DNase (QIAGEN K.K.) according to the manufacturer's instructions. For each sample, 500 ng of total RNA was reverse-transcribed using the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics K.K.) according to the manufacturer's instructions. The cDNA was diluted to 20% of its original concentration, and 5 μl of cDNA solution was used in a QRT-PCR (reaction mixture, 15 μl) with SYBR Premix Ex Taq (TaKaRa BIO Inc.) on a LightCycler system (Roche Diagnostics K.K.). The QRT-PCR products of *CsFTL1*, *CsFTL2*, and *CsFTL3* were cloned into the pGEM-T Easy Vector (Promega), and 10 clones were sequenced to verify the contributing genes. All the sequences were identical to those of *CsFTL1*, *CsFTL2*, and *CsFTL3*. The transcription levels of *CsFTL1*, *CsFTL2*, *CsFTL3*, *CsAFL1*, *CsSOC1*, *CsM111*, and *CsFL* in the samples were directly compared after normalization against the *CsACTIN* (AB679277) or *CsEF1α* (AB679278) loading standard. The 'calibrator sample' was designated as the most highly expressed time point for each gene of interest and therefore showed the highest relative expression level of 1.0 in each QRT-PCR run. The experiments were performed with three independently isolated RNA samples from the tissue. The data represent the mean ±standard error (SE) of at least three biological replicates. Primer sequences and PCR conditions are listed in Supplementary Table S1 (available at *JXB* online).

Arabidopsis and chrysanthemum transformation

A full-length coding sequence of *CsFTL3* was cloned into the vector pENTR221 (Invitrogen Carlsbad, CA, USA) via the BP reaction by using Gateway BP Clonase II enzyme mix (Invitrogen). The sequence was then subcloned into the binary vector pGWB2 (Nakagawa *et al.*, 2007) via the LR reaction by using Gateway LR Clonase II enzyme mix (Invitrogen) as described in the manufacturer's instructions. *Arabidopsis* (ecotype Columbia) plants were transformed using the floral dip method (Clough and Bent, 1998), and the flowering time of the T_3 line was evaluated. Transgenic lines of *C. morifolium* 'Jimba' were obtained by an *Agrobacterium*-mediated transformation system, as described by Aida *et al.* (2004). Candidate transformants were selected on the basis of paromomycin resistance. To confirm the presence of the transgene in T_0 plants, genomic DNA was extracted from the leaves and the *NPTII* region was amplified using *NPTII*-F (5′-GAGAGGCTATTCGGCTATGA-3′) and *NPTII*-R (5′-GATGCTCTTCGTCCAGATCA-3′) primers. Each T_0 line was propagated *in vitro*. The stem segments with axillary buds were used as explants. The emerged shoots were transplanted and used for further analysis.

Grafting of chrysanthemum plants

CsFTL3-overexpressing chrysanthemum plants (#20-66) and wild-type Jimba plants were used as stock. Wild-type Jimba plants (for experiment 1) and Nagano-queen plants (for experiment 2) were used as the scion, respectively. A wedge-shaped/slit grafting technique was applied, with the site of union wrapped with Parafilm. After grafting, the plants were kept for 2 weeks under high-humidity conditions, with a plastic film covering. In experiment 1, the plants were kept in a growth chamber at 20 °C with a 16 h photoperiod during the experiment (for 18 weeks). In experiment 2, the plants were kept in a closed greenhouse (maintained at an air temperature >18 °C, and ventilated when it rose above 25 °C) under a natural photoperiod plus a 6 h NB during the experiment (for 9 weeks).

Anatomical observations

For microscopic observation, tissue samples were fixed in formalin–acetic acid–alcohol (FAA) [70% ethanol:formalin:acetic acid, 90:5:5 (v/v/v)] and stored at room temperature. For observation by scanning electron microscopy (SEM), the fixed floral buds were dehydrated in an ethanol series [50, 70, 90, 95, and 99.5% (v/v)], following which ethanol was replaced with *t*-butanol. The samples were then freeze-dried and observed by SEM (VE-7000; Keyence Co., Osaka, Japan).

Results

Flowering response of C. seticuspe and identification of FT-like genes

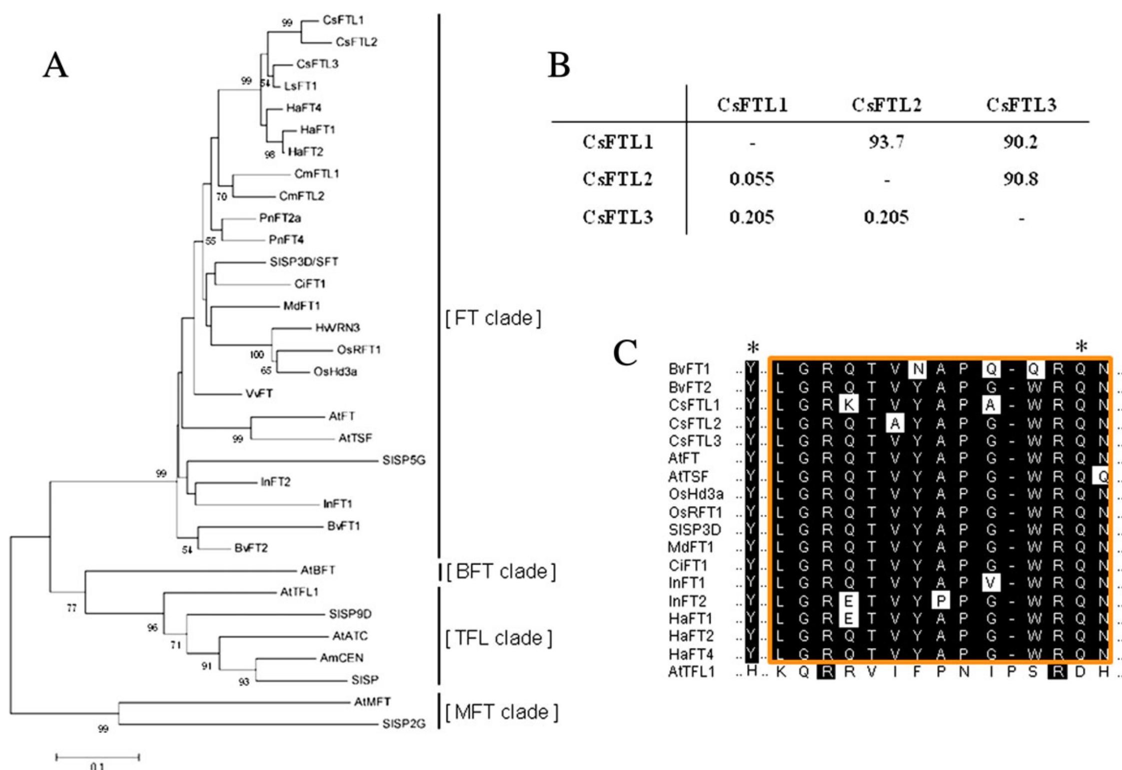
Flowering in *C. seticuspe* NIFS-3 was induced under 8 h SD conditions and efficiently inhibited under 16 h LD and 8 h SD+4 h NB conditions (Table 1). The number of days to visible flower bud formation was much less under floral-inductive SD conditions compared with that under non-inductive LD and NB conditions (Table 1).

Three *FT-like* genes, *CsFTL1* (AB679270), *CsFTL2* (AB679271), and *CsFTL3* (AB679272), which show significant homology to *FT/Hd3a*, were isolated from *C. seticuspe* NIFS-3. Sequence analysis of the deduced amino acid sequences showed that all the three *CsFTL* genes shared high identities (>90% at the amino acid sequence level) with each other (Fig. 1; Supplementary Fig. S1 at *JXB* online). The synonymous substitution rate between two sequences (i.e. K_s) provided a measure of time since divergence (Fig. 1). K_s for the comparison of *CsFTL3* with the other two *CsFTL* genes was 0.205, while that for the comparison of *CsFTL1* with *CsFTL2* was 0.055, indicating that duplication probably occurred within the genus *Chrysanthemum*. A phylogenetic tree was constructed using the amino acid sequences of several plant FT/Hd3a- and TFL1-like proteins (Fig. 1). The tree was divided into four major clades represented by FT, TFL1, BFT, and MFT. According to the phylogenetic tree analysis, all the three CsFTLs were clustered into the FT-like protein family and separated from the TFL1-, BFT-, and MFT-like protein families (Fig. 1). A single amino acid substitution of Tyr85 to His88 and of Gln140 to Asp144 is the most critical

Table 1. Effect of daylength on flowering in *C. seticuspe* NIFS-3

	Flowering (%)	Days to visible flower buds
SD	100	20.8±0.38
NB	0	>30
LD	0	>30

SD, short day (8 h photoperiod); NB, night break (8 h SD+4 h NB); LD, long day (16 h photoperiod).



	CsFTL1	CsFTL2	CsFTL3
CsFTL1	-	93.7	90.2
CsFTL2	0.055	-	90.8
CsFTL3	0.205	0.205	-

Fig. 1. Cloning of *FT-like* genes from *C. seticuspe*. (A) Phylogenetic tree based on the deduced amino acid sequences of the PEBP gene family, including those of *CsFTL1*, *CsFTL2*, and *CsFTL3*, from several plant species. The tree was constructed by the Neighbor–Joining method. Bootstrap percentages >50% are shown along the branches. Species abbreviations: *Antirrhinum majus*, Am; *Arabidopsis thaliana*, At; *Beta vulgaris*, Bv; *Citrus unshiu*, Ci; *Cucurbita maxima*, Cm; *Chrysanthemum seticuspe*, Cs; *Helianthus annuus*, Ha; *Hordeum vulgare*, Hv; *Ipomoea nil*, In; *Lactuca sativa*, Ls; *Malus×domestica*, Md; *Oryza sativa*, Os; *Populus nigra*, Pn; *Solanum lycopersicum*, Sl; and *Vitis vinifera*, Vv. (B) Comparisons of *CsFT* paralogues. K_s and amino acid sequence identity (%) values for pairwise comparisons of *CsFT* paralogues are shown: K_s values are shown below the diagonal, while amino acid sequence identity (%) values are shown above the diagonal. (C) Partial amino acid sequence alignment of the PEBP family members. Asterisks indicate the residues Tyr85(Y)/Gln140(Q) and His88(H)/Asp144(D) contributing to FT and TFL1 functioning, respectively (Hanzawa *et al.*, 2005; Ahn *et al.*, 2006). The conserved segment region B in the fourth exon, corresponding to the external loop of the PEBP family proteins, is boxed.

residue change for distinguishing FT and TFL1, respectively, in *Arabidopsis* (Hanzawa *et al.*, 2005; Ahn *et al.*, 2006). All the CsFTLs had Tyr84 and Gln139 in the positions corresponding to Tyr85 and Gln140, respectively, in *Arabidopsis* FT (Fig. 1; Supplementary Fig. S1). The segment B in the fourth exon, encoding an external loop of PEBP, is also important for the antagonistic functions of FT and TFL1 in *Arabidopsis* (Ahn *et al.*, 2006). In sugar beet, an antagonistic pair of FT paralogues, BvFT1 and BvFT2, have three substitutions in 14 amino acid residues in the external loop of PEBP, which is the major cause of their antagonistic functions (Pin *et al.*, 2010). The deduced amino acid sequence of CsFTL3 in the external loop of PEBP was identical to those of most other flowering-promoting FT-like proteins, including FT and BvFT2, whereas CsFTL1 and CsFTL2 had double and single amino acid substitutions, respectively, in the region (Fig. 1). CsFTL1 had the charge-changing substitution (lysine at the position Glu135 in BvFT2) and the replacement with a non-polar amino acid (alanine at the position Gly141 in BvFT2). These substitutions might have a profound effect on the activity of the FT-like proteins.

Organ-specific expression patterns of CsFTL1*,* CsFTL2*, and* CsFTL3 *in chrysanthemum*

To examine the organ-specific expression patterns of the three *CsFTL* genes in *C. seticuspe* at dawn, QRT-PCR analysis was performed. *CsFTL* mRNAs were detected in all the investigated tissues with huge differences in their expression levels (Fig. 2). The expression levels of all the three *CsFTL* genes were higher in the leaves than in the other tested tissues. Interestingly, the *CsFTL1* and *CsFTL2* expression levels were higher under NB (non-inductive) conditions with NB provided with red light than under SD (floral-inductive) conditions (Fig. 2). In contrast, the *CsFTL3* expression levels were higher under SD (floral-inductive) conditions than under non-inductive conditions (Fig. 2).

Expression patterns of CsFTL1*,* CsFTL2*, and* CsFTL3 *in chrysanthemum under different light conditions*

To investigate the effect of light on *CsFTL* expression in *C. seticuspe*, the relationship between the gene expression

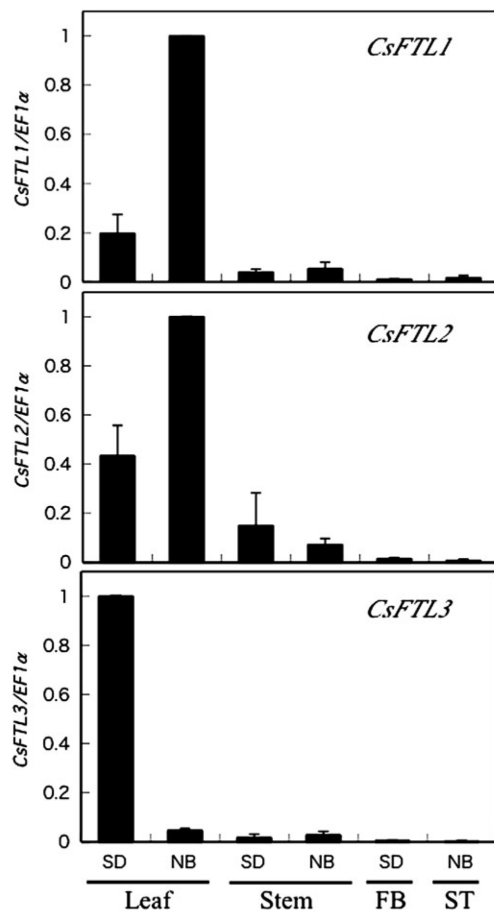


Fig. 2. Expression patterns of *CsFTL*s in different tissues of *C. seticuspe* under SD or NB conditions. *Chrysanthemum seticuspe* NIFS-3 plants were grown under LD (16 h) conditions and then transferred to SD (8 h) or NB (SD+15 min exposure to red light around the middle of the night) conditions. The tissues were harvested 4 weeks after the plants were transferred to SD or NB conditions and subjected to QRT-PCR analysis. FB, flower bud (~3 mm in diameter); ST, shoot tip (~3 mm long). *CsFTL1*, *CsFTL2*, and *CsFTL3* expression in the leaves was normalized against *CsEF1α* expression.

patterns in the leaves and floral-inductive photoperiod conditions was investigated. After 7 d of growth under LD conditions, the plants were shifted to SD conditions. The *CsFTL1* and *CsFTL2* transcript levels decreased after transfer to SD conditions (Fig. 3A), although *CsFTL2* expression was transiently increased 4 h after transfer to SD conditions under the experimental conditions. In contrast, the *CsFTL3* transcript levels rapidly increased after transfer to SD conditions; they were higher under SD conditions than under LD conditions in the dark period (Fig. 3A). Since *FT-like* genes, which accelerate flowering, are up-regulated under floral-inductive conditions (Kojima *et al.*, 2002; King *et al.*, 2006; Hayama *et al.*, 2007; Kikuchi *et al.*, 2009; Pin *et al.*, 2010), *CsFTL3* is selected as an *FT-like* gene candidate to regulate flowering in *C. seticuspe*. In addition, the counterparts of *CsFTL1*, *CsFTL2*, and *CsFTL3* were identified in a chrysanthemum cultivar, Reagan, and it was observed that their expression profiles in the Reagan plants exposed to LD or SD conditions for 7 d coincided with those in *C. seticuspe* under both LD and SD conditions (Supplementary Fig. S2 at *JXB* online). This suggests that the alternative model system of using *C. seticuspe* for chrysanthemum cultivars is applicable for basic phenomena in chrysanthemums, such as flowering.

Under repeated SD conditions, the expression of *CsFTL* genes in *C. seticuspe* plants showed diurnal oscillations (Fig. 3B). *CsFTL1* and *CsFTL2* transcripts accumulated during the dark period of the day, peaking around the middle of the dark period (Fig. 3B). Further, *CsFTL3* transcripts accumulated during the dark period of the day, peaking at dawn (Fig. 3B). Under repeated SD conditions, the *CsFTL3* transcript levels increased with SD cycles. The *CsFTL1* and *CsFTL2* transcript levels showed a clear diurnal oscillation as compared with the *CsFTL3* transcript levels. The diurnal oscillations suggested that the genes were under the control of the circadian clock. In fact, the *CsFTL1* transcript levels showed a circadian rhythm under both continuous light and continuous dark conditions, although the peak amplitude under continuous dark conditions was much lower than that under continuous light conditions (Fig. 3C). *CsFTL2* transcripts were barely detectable under continuous dark conditions but showed a similar pattern to *CsFTL1* transcripts under continuous light conditions (Fig. 3C). The *CsFTL3* transcript levels also showed a circadian rhythm under continuous dark conditions, peaking at subjective dawn; on the other hand, they showed an arrhythmic pattern under continuous light conditions (Fig. 3C). The transcript levels under the two different conditions (continuous light versus continuous darkness) were almost the same.

Comparison of CsFTL3 *expression and induction of floral integrator and/or floral identity genes,* CsAFL1*,* CsFL*, and* CsM111*, in the two accessions with different critical daylengths for flowering*

Chrysanthemum cultivars have different critical daylengths for flowering. The flowering response of two *C. seticuspe* accessions, NIFS-3 and Matsukawa, to different photoperiods (11, 12, 13, and 14 h) was examined. All NIFS-3 plants flowered under 11 h and 12 h photoperiods; photoperiods longer than 13 h completely suppressed flowering until the end of the experiment (Table 2). Leaf initiation increased dramatically and slightly before floral transition under 13 h and 12 h photoperiods, respectively, compared with that under an 11 h photoperiod (Table 2). All Matsukawa plants flowered under 11, 12, and 13 h photoperiods; a 14 h photoperiod completely suppressed flowering until the end of the experiment (Table 2). Leaf initiation increased dramatically and slightly before floral transition under 14 h and 13 h photoperiods, respectively, compared with that under 11 h and 12 h photoperiods (Table 2). Therefore, NIFS-3 and Matsukawa are estimated to have 12 h and 13 h photoperiods as their critical daylengths for flowering.

The *CsFTL3* expression levels were investigated in both NIFS-3 and Matsukawa plants grown under the different photoperiod conditions described above. Leaves from the

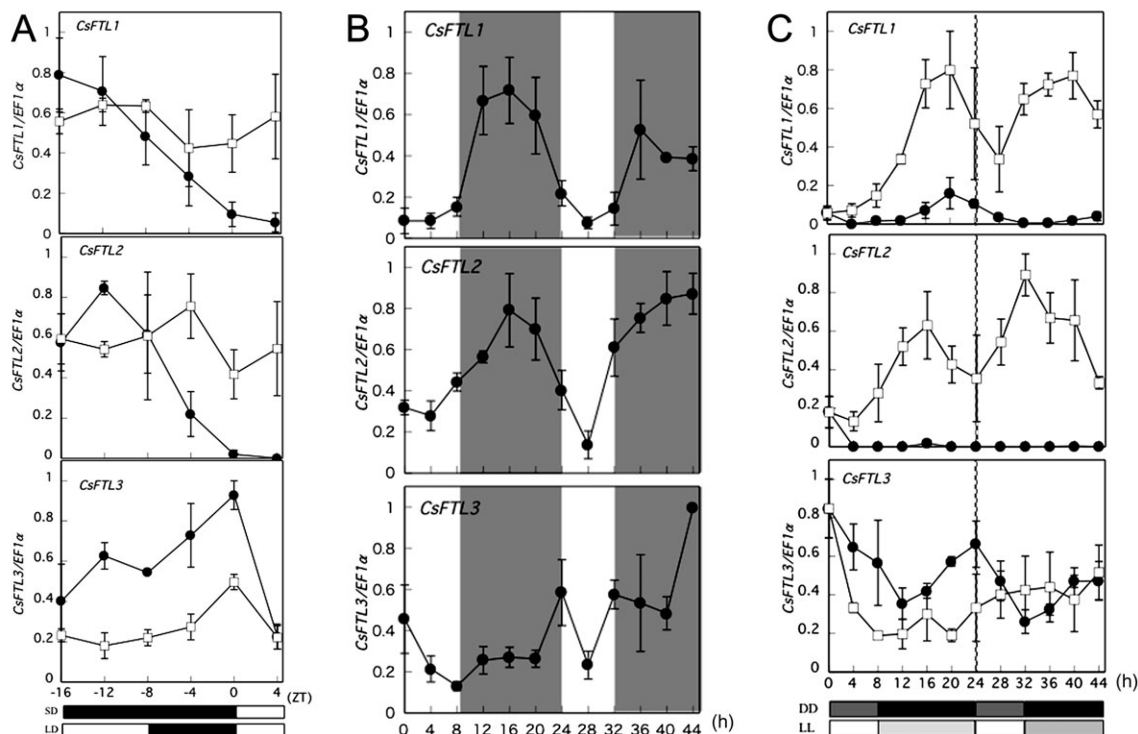


Fig. 3. Expression patterns of *CsFTL*s in leaves of *C. seticuspe* under different light conditions. (A) Diurnal expression patterns of *CsFTL* genes in *C. seticuspe* under LD conditions and after transfer to SD conditions. *Chrysanthemum seticuspe* NIFS-3 plants were grown under 16 h LD conditions and then transferred to 8 h SD (filled circles) or maintained under 16 h LD (open squares) conditions. White and black horizontal bars represent light and dark periods, respectively. (B) Diurnal expression patterns of *CsFTL* genes in *C. seticuspe* under repeated SD conditions. *Chrysanthemum seticuspe* NIFS-3 plants were transferred to 8 h SD conditions after growth under 16 h LD conditions. Transcription levels were monitored from the beginning of the second SD cycle for the subsequent 44 h. Grey vertical areas represent dark periods. (C) Circadian expression patterns of *CsFTL* genes in *C. seticuspe* under continuous dark and continuous light conditions. *Chrysanthemum seticuspe* NIFS-3 plants exposed to an 8 h SD cycle after growth under 16 h LD conditions were transferred to continuous dark (DD; filled circles) or continuous light (LL; open squares) conditions. Transcription levels were monitored for the subsequent 44 h. All expression values of *CsFTL1*, *CsFTL2*, and *CsFTL3* were normalized against *CsEF1α* expression.

Table 2. Flowering response of *C. seticuspe* NIFS-3 and Matsukawa under different daylengths

Daylength (h)	NIFS-3		Matsukawa	
	Flowering (%)	No. of leaves	Flowering (%)	No. of leaves
11	100	21.4±1.43	100	20.4±1.17
12	100	23.9±2.18	100	21.8±0.79
13	0	>30	100	24.7±0.82
14	0	>30	0	>30

plants already exposed to seven cycles of each photoperiod were collected every 4 h over a 20 h period. The *CsFTL3* expression levels increased as the photoperiod shortened (Fig. 4A). The *CsFTL3* expression levels in NIFS-3 remained low under photoperiods >13 h, compared with those under 11 h and 12 h photoperiods; however, the expression levels in Matsukawa remained low only under a 14 h photoperiod (Fig. 4A). These *CsFTL3* expression patterns are correlated with their estimated critical daylengths for flowering. The *CsFTL3* mRNA expression levels clearly exhibited a diurnal rhythm in plants grown under shorter photoperiods; they started to increase towards the end of the night and peaked by midday (Fig. 4A).

The expression of floral integrator and/or floral identity genes in shoot apexes under the different daylength conditions described above was also investigated. The *C. seticuspe* counterparts of *CmAFL1*, *CmFL*, and *CDM111* (Shchennikova *et al.*, 2004; Li *et al.*, 2009) were cloned and named *CsAFL1* (AB679273), *CsFL* (AB679274), and *CsM111* (AB679275), respectively. The coding sequences of *CsAFL1*, *CsFL*, and *CsM111* cDNAs exhibited 99.4, 99.8, and 99.7% identity, respectively, to those of their counterparts at the nucleotide level. The expression of these floral integrator and/or floral identity genes was examined in shoot apexes every 4 d over a 20 d period. *CsAFL1*, *CsFL*, and *CsM111* were involved in the photoperiodic regulation of floral transition (Fig. 4B). In NIFS-3, the *CsAFL1* expression levels started to increase 4 d after the plants were transferred to 11 h and 12 h photoperiod conditions; they increased more rapidly after day 4 (Fig. 4B). Although the *CsAFL1* transcript levels under a 13 h photoperiod were lower than those under 11 h and 12 h photoperiods, *CsAFL1* expression

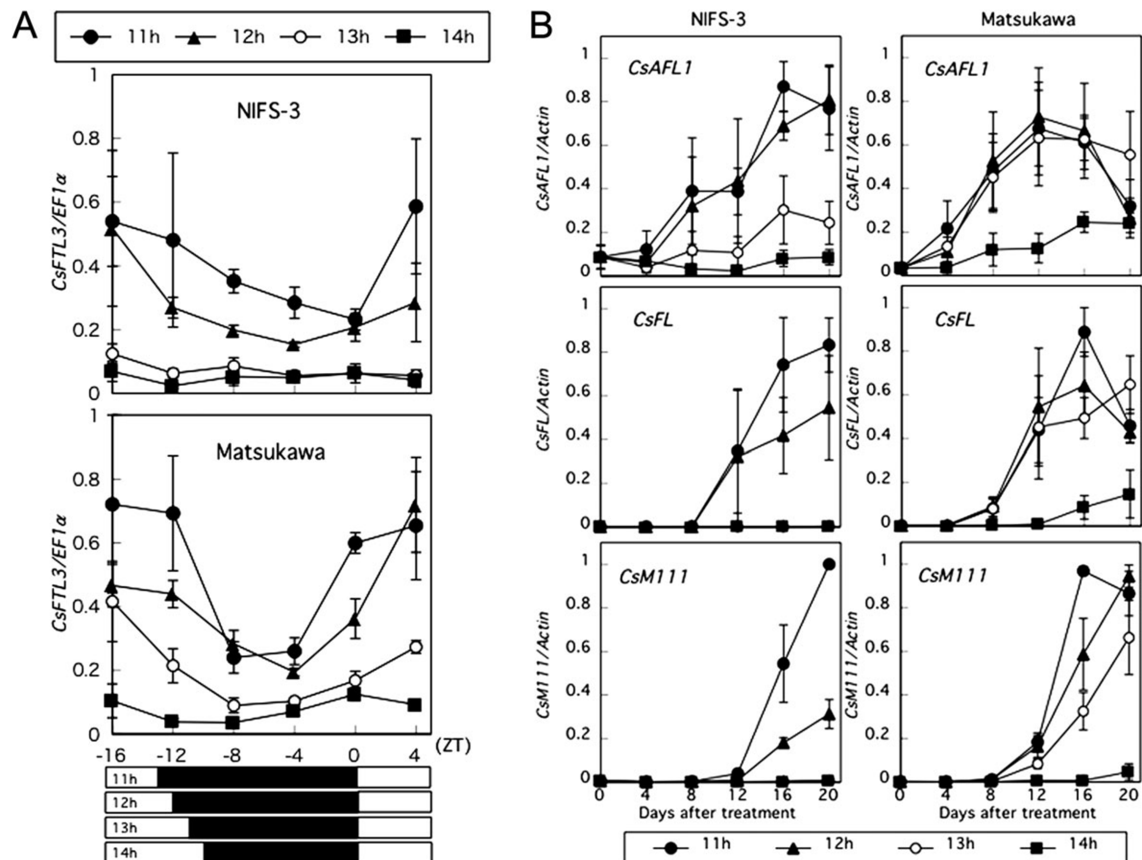


Fig. 4. Analysis of *CsFTL3* in leaves and floral integrator and/or floral identity gene expression in shoot tips in two different *C. seticuspe* accessions under different daylengths. Two different *C. seticuspe* accessions, NIFS-3 and Matsukawa, were grown under 16 h LD conditions and then transferred to 11 h (filled circles), 12 h (filled triangles), 13 h (open circles), or 14 h (filled squares) photoperiod conditions. (A) Diurnal expression patterns of *CsFTL3* in leaves. Seven days after transfer, leaves were harvested every 4 h and subjected to QRT-PCR analysis. The *CsFTL3* expression was normalized against *CsEF1α* expression. White and black horizontal bars represent light and dark periods, respectively. (B) Analysis of floral integrator and/or floral identity gene expression in shoot tips. Shoot apexes were harvested every 4 d over a 20 d period and subjected to QRT-PCR analysis. The expression of each gene of interest was normalized against *CsACTIN* expression.

increased slightly over the 20 d period under the 13 h photoperiod. The *CsAFL1* expression levels remained low under a 14 h photoperiod. *CsFL* and *CsM111* transcripts were first detected on day 12 under 11 h and 12 h photoperiods; the increase in the *CsFL* and *CsM111* expression levels was higher under an 11 h photoperiod than under a 12 h photoperiod (Fig. 4B). In Matsukawa, the *CsAFL1* expression levels started to increase 4 d after the plants were transferred to 11, 12, and 13 h photoperiod conditions; they increased more rapidly after day 4 (Fig. 4B). *CsAFL1* expression decreased slightly on day 20 under 11 h and 12 h photoperiods. On day 20, the *CsAFL1* transcript levels under 11 h and 12 h photoperiods were the same as those under a 14 h photoperiod. *CsFL* transcripts were first detected on day 8 under 11, 12, and 13 h photoperiods and on day 16 under a 14 h photoperiod (Fig. 4B). Under 11, 12, and 13 h photoperiods, *CsM111* transcripts were first detected on day 12; their levels increased more rapidly after day 16 under shorter photoperiods (Fig. 4B). Under a 14 h photoperiod, the *CsM111* expression levels remained relatively low. Thus, *CsAFL1*, *CsFL*, and *CsM111* were coordinately activated after floral transition in shoot apexes and their induction closely coincided with the accessions' critical daylengths for flowering (Table 2).

Early flowering due to the ectopic expression of CsFTL3 *in chrysanthemum and* Arabidopsis

To investigate the potential function of *CsFTL3* as a floral promoter, transgenic experiments with chrysanthemum were performed. Transgenic chrysanthemum plants with *CsFTL3* expressed under the control of the *Cauliflower mosaic virus* (CaMV) *35S* promoter were generated. As a result, 30 independent T_0 lines were obtained and their *FTL3* (*CsFTL3* and *CmFTL3*) expression levels were investigated. Of these, 14 transgenic lines of *in vitro* plants developed flower buds under non-inductive LD conditions, with high *FTL3* transcript levels (Supplementary Fig. S3 at *JXB* online). Six transgenic lines were selected on the basis of the preliminary analysis of exogenous *CsFTL3* mRNA levels. Depending on soil conditions, three out of the six transgenic lines (# 20-66, #19-12, and #20-89) flowered

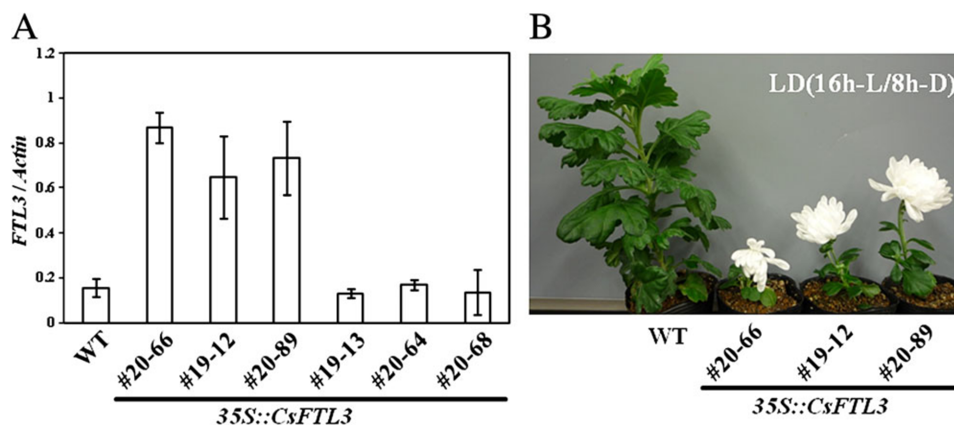


Fig. 5. Effect of *CsFTL3* overexpression on flowering in *Chrysanthemum morifolium* under LD conditions. (A) Transcript levels of *FTL3* (*CsFTL3* and *CmFTL3*) in the leaves were analysed by QRT-PCR in six independent transgenic lines and wild-type (WT) plants. (B) Three independent transgenic lines showed high expression levels of *FTL3* and flowered under LD conditions, while the wild-type plants did not.

under non-inductive LD conditions (16 h), with 5–7 times higher *FTL3* mRNA levels than those in the wild-type plants (Fig. 5A, B). The other three out of the six transgenic lines (#19-13, #20-64, and #20-68) did not show any visible flower buds under LD conditions or any alterations in their *FTL3* mRNA levels compared with those in the wild-type plants (Fig. 5A). Furthermore, transgenic *Arabidopsis*, which ectopically expressed *CsFTL3*, flowered earlier than the wild-type plants under SD conditions (Supplementary Fig. S4 at *JXB* online).

To demonstrate the potential function of the gene product of *CsFTL3* as a graft-transmissible floral promoter, florigen, in chrysanthemum, grafting experiments were performed with *CsFTL3*-overexpressing transgenic plants (#20-66). The experiments showed the translocation of CsFTL3 from the transgenic stock to the wild-type scion under non-inductive LD or NB conditions. Four out of six plants with Jimba (experiment 1) and all the plants with Nagano-queen (experiment 2) resulting from grafting between the wild-type and *35S::CsFTL3* transgenic plants produced a crown bud, a flower bud that initiates capitulum primordia with involucral bracts but in which the development is arrested before floret initiation (Fig. 6; Supplementary Table S2 at *JXB* online). It is a sign of the transition to the reproductive phase in chrysanthemums. All the plants resulting from grafting between the wild-type stock and scion remained vegetative. To induce transfer of the floral stimulus from the donor to the receptor in grafting experiments, it is often necessary to defoliate the receptor plants (Thomas and Vince-Prue, 1997). In this study, defoliating the receptor plants increased the number of plants producing a crown bud (Supplementary Table S2).

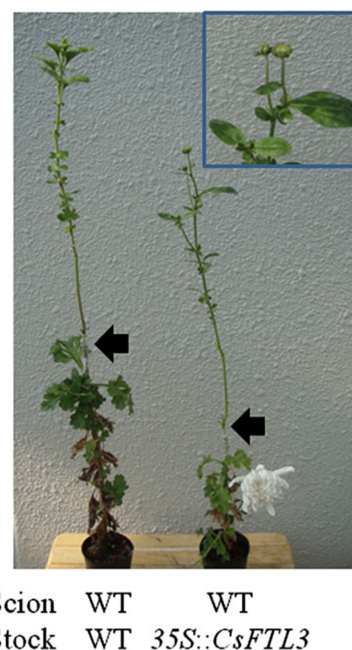


Fig. 6. Grafting experiment with *Chrysanthemum morifolium* plants overexpressing *CsFTL3* under NB conditions. Plants resulting from grafting between the Nagano-queen wild-type plants (WT) and *35S::CsFTL3* transgenic plants (#20-66) produced a crown bud, a flower bud that initiates the capitulum primordia with involucral bracts. Arrows indicate the graft junctions. Photograph was taken at 9 weeks after the grafting.

Number of SD cycles required for the initiation and development of capitulum in chrysanthemums

The NIFS-3 plants maintained under NB conditions (8 h SD+4 h NB with fluorescent tubes) continued their vegetative growth for 70 d, whereas those maintained under SD conditions flowered by 50 d from the initiation of the SD cycle (Fig. 7). The plants required four SD cycles to determine the node number below the terminal capitulum. The first morphological signs of the initiation of capitulum primordia and involucral scales (bracts or modified leaves) appeared at the periphery of the dome on day 8; floret primordia differentiated acropetally from the bottom of the capitulum meristem on day 12. About 50% of the dome of the enlarged capitulum meristem was covered with floret

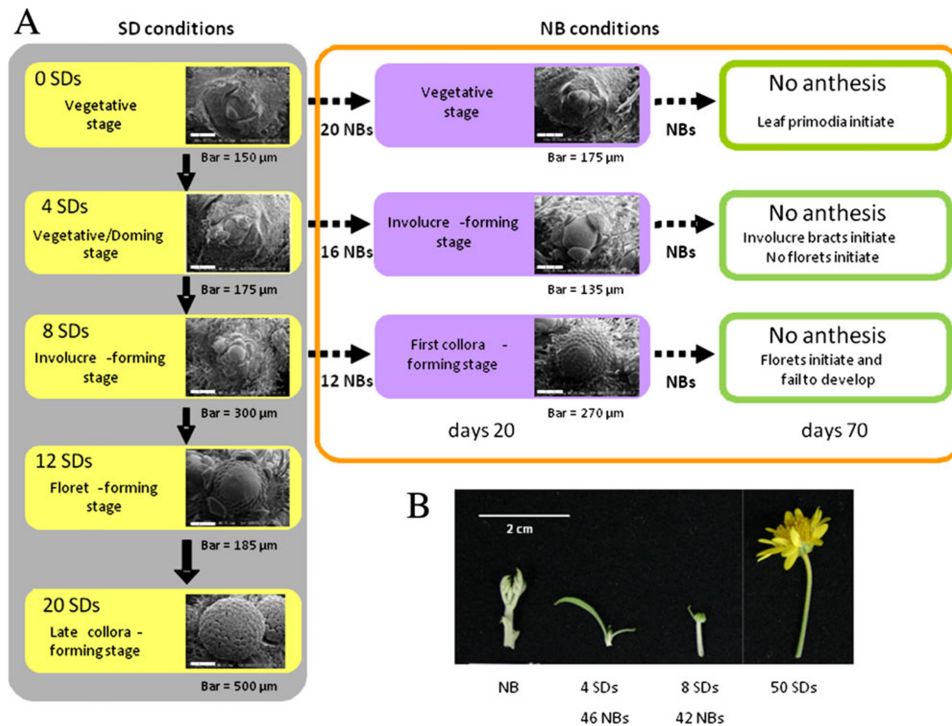


Fig. 7. Alternative pathways of capitulum development in *C. seticuspe*. *Chrysanthemum seticuspe* NIFS-3 plants were grown under NB conditions (8 h SD+4 h NB) and then subjected to different treatments: maintained under NB conditions (0 d of SD exposure); transferred to 8 h SD conditions (4 d or 8 d) and then returned to NB conditions; or maintained under SD conditions. (A) SEM images illustrating the developmental stages of the capitulum in *C. seticuspe* under 8 h SD conditions and the change in development on transfer to NB conditions. (B) The photograph was taken at 50 d after the initiation of the SD cycle.

primordia (Fig. 7). The development changed on transfer to NB conditions. The plants transferred to NB conditions after exposure to SD conditions for 4 d produced a crown bud, which initiated involucral bracts on the edge of the apical receptacle; however, the plants did not initiate florets on the apical receptacle until the end of the experiment (day 70). The plants transferred to NB conditions after exposure to SD conditions for 8 d initiated florets on the apical receptacle, but capitulum development was strongly suppressed by NB conditions.

Alteration in the photoperiodic cycle (i.e. transfer of plants from SD to NB conditions) down-regulated *FTL3* expression in the leaves (Supplementary Fig. S5 at *JXB* online). The expression of the floral integrator and/or floral identity genes *CsAFL1*, *CsSOC1* (AB679276), *CsFL*, and *CsM111* was examined in shoot apexes every 4 d. Under NB conditions, the expression levels of all genes of interest remained low or undetectable (Fig. 8). The expression patterns of *CsAFL1*, *CsFL*, and *CsM111* in the plants maintained under 8 h SD conditions were identical to those in the plants grown under an 11 h photoperiod (Figs 4B, 8). *CsSOC1* expression in the plants maintained under SD conditions increased, peaking on day 8; after day 8, the expression levels started to decrease gradually, almost reaching the levels observed initially (Fig. 8). In the plants exposed to SD conditions for 4 d, the expression levels of all genes of interest increased slightly as compared with those in the plants maintained under NB conditions, but remained low at all tested time points (Fig. 8). In the plants exposed to SD conditions for 8 d, *CsAFL1* and *CsSOC1* expression was suppressed after transfer to NB conditions (Fig. 8). On the other hand, the expression levels of *CsFL* and *CsM111* were identical to those in the plants maintained under SD conditions until day 12, but were suppressed thereafter (Fig. 8).

Discussion

Chrysanthemum has been used in classical physiological experiments on flowering. These experiments have demonstrated the presence of a kind of hormonal substance, called a florigen, produced in the leaves (for a review, see Chailakhyan and Krikorian, 1975). The present study used *C. seticuspe*, a wild diploid chrysanthemum. Under the present experimental conditions, two accessions of *C. seticuspe*, NIFS-3 and Matsukawa, showed typical photoperiodic flowering responses as obligate SD plants (Tables 1, 2). The *FT-like* genes *CsFTL1*, *CsFTL2*, and *CsFTL3* and the floral integrator and/or floral identity genes *CsAFL1*, *CsSOC1*, *CsFL*, and *CsM111* isolated from *C. seticuspe* showed high sequence identity (>98.8%) to their *C. morifolium* counterparts. The expression profiles of the *FT-like* and floral integrator and/or floral identity genes in *C. morifolium* coincided with those in *C. seticuspe* under both

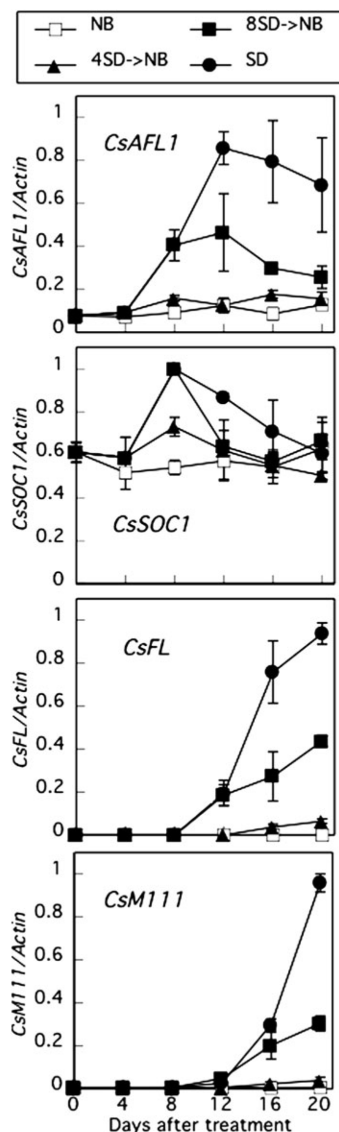


Fig. 8. Effect of transfer from SD to NB conditions on the floral integrator and/or floral identity gene expression in *C. seticuspe*. *Chrysanthemum seticuspe* NIFS-3 plants were grown as described in Fig. 7. The plants were maintained under NB conditions (0 d of SD exposure; open squares); exposed to SD conditions for 4 d (filled triangles) or 8 d (filled squares) and then transferred to NB conditions; or maintained under SD conditions (filled circles). Shoot apexes were harvested every 4 d over a 20 d period and subjected to QRT-PCR analysis. The expression of each gene of interest was normalized against *CsACTIN* expression.

SD and LD conditions (Figs 3A, 4A; Li *et al.*, 2009). These observations suggest that to understand the genetic and molecular mechanisms of the reproductive process in chrysanthemums and overcome the issue of complex hybridity and polyploidy, *C. seticuspe* can be a useful alternative model of chrysanthemum cultivars.

Identification of FT-like *genes in chrysanthemum*

In this study, *CsFTL1*, *CsFTL2* and *CsFTL3* were isolated as *FT-like* genes from *C. seticuspe*. These chrysanthemum paralogues of *Arabidopsis FT* are more closely related to each other than to homologues from other species (Fig. 1). The synonymous substitution rate between two sequences (i.e. K_s) and phylogenetic analysis indicated that *CsFTL3* is separated from *CsFTL1* and *CsFTL2* (Fig. 1). Amino acid sequence comparison of the three encoded proteins showed that all the three CsFTLs carried the functionally important signatures Tyr84 and Gln139 in the positions corresponding to Tyr85 and Gln140, respectively, in *Arabidopsis* FT (Fig. 1; Supplementary Fig. S1 at *JXB* online), which are important for the antagonistic functions of FT and TFL1, respectively, in *Arabidopsis* (Hanzawa *et al.*, 2005; Ahn *et al.*, 2006). Unlike two *FT* family genes in *Arabidopsis*, *FT* and *TSF*, which exhibit similar expression patterns, the *FT-like* genes in chrysanthemum showed distinctly different expression patterns. All the three *FT-like* genes were expressed mainly in the leaves, as expected (Fig. 2). *CsFTL3* was up-regulated under floral-inductive SD conditions, whereas *CsFTL1* and *CsFTL2* were down-regulated, showing an antagonistic expression pattern to that of *CsFTL3*; *CsFTL1* and *CsFTL2* were preferentially expressed under non-inductive conditions (Figs 2, 3A). The study showed a particularly strong correlation between flowering and *CsFTL3* expression under various photoperiodic conditions in the two *C. seticuspe* accessions that differed in their critical daylengths for flowering (Table 2; Fig. 4A). Recently, a negative effect of *FT-like* genes on flowering has been postulated in sugar beet (Pin *et al.*, 2010) and sunflower (Blackman *et al.*, 2010). In sugar beet, amino acid changes within the region encoding an external loop of PEBP determine the ability of BvFT2 and BvFT1 to promote and repress flowering, respectively (Pin *et al.*, 2010). CsFTL3 has the amino acids in the external loop of PEBP which are conserved in most other FT-like proteins, whereas CsFTL1 and CsFTL2 had substituted amino acids in the region (Fig. 1). Correlation between flowering and the expression patterns of the three *CsFTL* genes together with sequence comparison indicated that at least *CsFTL3* might have a hypothetical function as a mobile signal for flower induction under SD conditions, and the other closely related *FT-like* genes in *C. seticuspe* (i.e. *CsFTL1* and *CsFTL2*) might have distinct roles as transmissible signals, possibly antagonistic functions as postulated in sugar beet and sunflower.

Early flowering due to the ectopic expression of CsFTL3 *in* Arabidopsis *and chrysanthemum*

Transgenic *Arabidopsis* plants overexpressing *CsFTL3* flowered earlier than the wild-type plants (Supplementary Fig. S4 at *JXB* online). Early flowering phenotypes of transgenic *Arabidopsis* are reported to occur upon ectopic expression of *FT-like* genes from heterologous plant species such as tomatoes (Lifschitz *et al.*, 2006) and poplars (Igasaki *et al.*, 2008). This shows that *CsFTL3* has the potential to induce early flowering in *Arabidopsis* in a similar manner to that of the *FT-like* genes in other plant species. In functional complementation analyses using *Arabidopsis*, transgenic plants overexpressing *FT-like* genes isolated from

lettuce and sunflower, and which showed close correlation with their chrysanthemum paralogues in phylogenetic analysis, did not flower as early as the plants overexpressing *Arabidopsis FT* (Blackman *et al.*, 2010; Fukuda *et al.*, 2011). The extremely early flowering phenotype of *CsFTL3*-overexpressing *Arabidopsis* plants could not be observed in this study (Supplementary Fig. S4). This suggests that some functional difference exists between FT in *Arabidopsis* and that in Compositae species such as chrysanthemum, lettuce, and sunflower.

To investigate the potential function of CsFTL3 as a floral promoter in chrysanthemum, transgenic experiments with chrysanthemum were performed. The transgenic chrysanthemum plants with high *CsFTL3* expression levels (#20-66, #19-12, and #20-89) flowered *in vitro* and in soil under non-inductive LD conditions (Fig. 5; Supplementary Fig. S3 at *JXB* online). These results strongly indicated that the gene product of *CsFTL3* could function as a floral activator to regulate flowering under non-inductive LD conditions. Post-transcriptional regulation might have altered the level of protein production by *CsFTL3* in the experiment. Aida *et al.* (2008) reported that the β-glucuronidase (GUS) activity levels in their study did not quantitatively correspond to the GUS mRNA expression levels, but depended on the translational efficiency of the transgenes in a GUS assay of chrysanthemum transformants. This is a potential reason why some of the transgenic plants with high *CsFTL3* expression levels did not show the early flowering phenotype (Supplementary Fig. S3).

The grafting experiment with *CsFTL3*-overexpressing chrysanthemum plants (#20-66) may reflect the translocation of CsFTL3 from the transgenic stock to the wild-type scion under non-inductive LD conditions, although the plants failed to flower (Fig. 6). The reason might be that not enough graft-transmissible signals were transmitted to the wild-type scion for complete development of the capitulum. This small effect may be explained in terms of the sink–source relationship between the receptor shoot and the donor stock (Zeevaart, 2006). There is another argument that transmissible inhibitors may be produced in the leaves when the plants are exposed to non-inductive photoperiodic cycles (Lang *et al.*, 1977; Thomas and Vince-Prue, 1997). The result suggested that the gene product of *CsFTL3* could function as a graft-transmissible signal, florigen, in chrysanthemum, and that there possibly exists an antagonistic inhibitor in the wild-type scion under non-inductive LD conditions.

Correlation between CsFTL3 *expression in leaves and critical daylengths for flowering*

Elucidation of the mechanisms underlying the determination of critical daylengths for flowering will offer the advantage of year-round chrysanthemum production. In *Pharbitis* (*Ipomoea*) *nil* (L.) Choisy (Hayama *et al.*, 2007) and rice (Itoh *et al.*, 2010), orthologues of the *FT* gene are highly expressed when the daylength is shorter than their respective critical daylengths for flowering; conversely, the expression levels decrease markedly as the photoperiod increases. In this study, differences were observed in critical daylengths for flowering and *CsFTL3* expression between the *C. seticuspe* accessions NIFS-3 (<12 h) and Matsukawa (<13 h) (Fig. 4A; Table 2). Consistent with the increased *CsFTL3* mRNA expression, the expression of the floral activator genes *CsAFL1*, *CsFL*, and *CsM111* was up-regulated in shoot tips under shorter photoperiods (Fig. 4B). Together with the function of *CsFTL3* as a floral inducer, increased *CsFTL3* expression in leaves seems to be a key step in the gene activation cascades involved in the induction of flowering in *C. seticuspe* under SD conditions. The mRNA expression of orthologues of the *FT* gene, namely *PnFT* in *P. nil* and *Hd3a* in rice, is suppressed to undetectable levels when daylength is longer than the critical daylengths for flowering (Hayama *et al.*, 2007; Itoh *et al.*, 2010). In contrast, detectable expression levels of *CsFTL3* transcripts were observed when daylength was longer than the critical daylengths in both the *C. seticuspe* accessions; they were relatively low compared with those under floral-inductive conditions (Figs 3A, 4A). This might indicate that chrysanthemums require a threshold expression level of *CsFTL3* mRNA in leaves for flowering. As argued, perhaps, the fact also indicates the possibility of the existence of an antagonistic inhibitor in unfavourable photoperiodic cycles, which suppresses the function of the gene product of *CsFTL3* as a floral inducer.

Effect of light period on and circadian regulation of the expression of FT-like *genes*

It has been demonstrated that a period of light preceding an inductive dark period is essential for flowering in chrysanthemums: a minimum of 3–5 h of light per day is required (for a review, see Cathey, 1969). In the present study, the expression of *CsFTL3*, which is involved in flowering, increased during repeated 8 h SD cycles (Fig. 3B). However, the expression did not increase under continuous dark conditions (Fig. 3C). This indicates that a period of light preceding an inductive dark period is important for *CsFTL3* expression, and, thus, flowering. In addition, there is a clear relationship between the daily light integral during SDs and the time to both flower initiation and flower development in chrysanthemums (for a review, see Carvalho and Heuvelink, 2001). Low light intensity suppresses floral transition under floral-inductive SD conditions. Although it is not clear whether the daily light integral regulates *CsFTL3* expression, in *Arabidopsis* LD promotion of flowering with FT expression up-regulated by 'photosynthetically active radiation'—possibly mediated by sucrose—has been highlighted (King *et al.*, 2008).

Recent advances in molecular biology have elucidated the mechanisms underlying the determination of the accurate critical daylength threshold. In the external coincidence model (Pittendrigh and Minis, 1964), a signal is produced when an environmental signal (light) coincides with the sensitive phase of an endogenous circadian rhythm of photoresponsiveness. Two distinct gating mechanisms in rice could enable the manipulation of slight differences in

daylength to control *Hd3a* transcription with a critical daylength threshold (Itoh *et al.*, 2010). In the present study, expression of *CsFTL* genes showed diurnal oscillations (Fig. 3B) and a circadian rhythm after the plants were exposed to an SD cycle (Fig. 3C). A key challenge now is to determine how the divergent photoperiodic flowering responses are regulated in chrysanthemums via the control of the genes regulated by both light and the circadian clock. *CsFTL* genes will provide important information with recent advances in the understanding of the mechanisms underlying the determination of the accurate critical daylength threshold.

Gene activation cascade for flowering under SD conditions in chrysanthemums

The SAM generates leaves and shoots during the vegetative phase; in the reproductive phase, after floral transition, it becomes transformed into an inflorescence meristem, and the new lateral primordia produced develop as floral meristems. The chrysanthemum flower is a capitulum, a head type of inflorescence, which apparently mimics a large single flower, with the elongated ray florets located on the edge of the receptacle and the disc florets located at the centre of the receptacle. The photoperiodicity for the development of the capitulum as a large single flower in chrysanthemums differs from that for floral initiation and developmental processes in other model plants. *Chrysanthemum seticuspe* plants require SD cycles for capitulum development (Fig. 7). In *Arabidopsis*, *FT* activation in leaves induces the transition to the reproductive phase and flowering. The FT protein translocates into the SAM and interacts with FD, forming the FT–FD complex, which regulates the downstream target genes such as *AP1* and *FUL* (Abe *et al.*, 2005; Wigge *et al.*, 2005). It has been indicated that *AP1* expression is a relatively late event during floral induction. In *Arabidopsis*, *LFY* and *FUL* [*AGAMOUS-LIKE 8* (*AGL8*)] are induced 24 h after plants are transferred to floral-inductive LD conditions, while *AP1* is induced 72 h after transfer (Hempel *et al.*, 1997). It has been suggested that SOC1 mainly regulates *LFY* for floral initiation in the SAM (Lee *et al.*, 2008). SOC1 and LFY at the SAM also influence phase transition through the regulation of the GA pathway (Lee and Lee, 2010). *LFY* and *AP1* are the key regulators of floral meristem identity and the main activators of the cascade of genes initiating floral development. In the two *C. seticuspe* accessions that differed in their critical daylengths for flowering, the expression levels of *CsFTL3* in the leaves closely coincided with the induction of floral integrator and/or floral identity genes and the accessions' flowering responses (Figs 3A, 4A; Tables 1, 2). In *Arabidopsis*, *FUL* is expressed throughout the shoot apex and the expression is up-regulated in inflorescence meristems, consistent with floral transition and identity (Ferrandiz *et al.*, 2000). The expression patterns of *CsAFL1*, a homologue of *FUL*, suggest that *CsAFL1* functions around the time of the transition to the reproductive phase and during early capitulum development in *C. seticuspe*, similar to *FUL* in *Arabidopsis*. It has been suggested that *CO* activates *SOC1* mainly through the regulation of *FT*, and up-regulation of *SOC1* in the SAM is one of the earliest events in floral transition (Lee and Lee, 2010). In the SAM, interaction of SOC1 with AGL24 induces *LFY* expression (Lee *et al.*, 2008). In *C. seticuspe*, *CsSOC1* expression in the shoot apexes was up-regulated at the time of the first morphological signs of capitulum primordia initiation (day 8; Figs 7, 8). After the up-regulation of *CsSOC1* expression, *CsFL*, an *LFY* homologue, was induced in the shoot apexes (day 12; Fig. 8). *CsSOC1* expression was not up-regulated and *CsFL* was not induced in the plants maintained under NB conditions (Fig. 8). No florets were initiated and *CsFL* was only weakly expressed with slightly up-regulated *CsSOC1* expression in the plants exposed to SD conditions for 4 d (Figs 7, 8). It seems that *CsFL* induction by CsSOC1, possibly like *LFY* induction by SOC1 through interaction with AGL24, is conserved in the process of floral meristem induction in *C. seticuspe*. *CsFL* and *CsM111*, an *AP1* homologue, were induced—*CsFL* slightly earlier than *CsM111*—at the time of the first morphological signs of floret primordia initiation (day 12; Figs 7, 8). The expression patterns of *CsFL* and *CsM111* suggest that they function around the time of floral meristem (floret) differentiation rather than at the time of the transition to the reproductive phase. In *Arabidopsis*, shortly after the onset of floret differentiation, *SOC1* is down-regulated by AP1 to initiate the downstream pathways required for inducing B- and C-class floral organ identity genes (Liu *et al.*, 2008). As in *Arabidopsis*, it was observed in *C. seticuspe* plants maintained under SD conditions that temporally increased *CsSOC1* expression started to decrease with *CsM111* induction and floret initiation (Figs 7, 8). The present study results suggest that floral integrator and/or floral identity gene activation cascades are conserved in the floral transition and developmental processes regulated by photoperiodic cycles in *C. seticuspe*, like in *Arabidopsis*.

The approach of reciprocal transfer experiments was useful for further investigation of the activation cascades involved in floral transition and development regulated by photoperiodic cycles in chrysanthemums. In plants that have been studied in detail as physiological models of SD or LD plants, such as *P. nil* (Imamura, 1967), *Sinapis alba* L. (Bernier, 1963), *Lolium temulentum* L. (Evans, 1958), and *Arabidopsis* (Hempel and Feldman, 1994), floral transition can be induced after exposure to a single inductive photoperiod. When floral transition is induced by a single inductive photoperiod, the floral primordia proceed to develop further under non-inductive conditions. However, chrysanthemums require SD cycles for capitulum and floret development; non-inductive conditions inhibit this development (Cockshull and Horridge, 1980; Adams *et al.*, 1998). In the SD plant *P. nil*, *PnFT* abundance induced by a single floral-inductive dark period is sufficient for flower bud initiation and development (Hayama *et al.*, 2007; Higuchi *et al.*, 2011). In the LD plant *S. alba*, induction of flowering by a single photoperiodic cycle is fine-tuned by *SaFT* in the leaves (D'Aloia *et al.*, 2009). *SaFT* abundance induced by

a single floral-inductive light period is sufficient for flowering. In the SAM, *SaSOC1*, *SaLFY*, and *SaAP1* activation occurs after *SaFT* activation in the leaves. *SaAP1* activation is considered the final step in floral transition in the SAM. A similar activation pattern for *FT* in leaves is observed on the induction of flowering by a single photoperiodic cycle in *Arabidopsis* (King *et al.*, 2008). Unlike the above-mentioned model plants, *C. seticuspe* plants require SD cycles for flowering (i.e. capitulum development; Fig. 7). In the *C. seticuspe* plants exposed to SD conditions for 4 d, the node number below the terminal capitulum was determined and a crown bud was produced (Fig. 7). This response indicated that a slight increase in the *CsAFL1* and *CsSOC1* transcript levels could induce the transition to the reproductive phase (Fig. 8). In the *C. seticuspe* plants exposed to SD conditions for 8 d, the initiated florets failed to develop and the expression of all the tested genes was remarkably suppressed after transfer to NB conditions (Figs 7, 8). This indicated that the activities of CsFL and CsM111 in the plants were not sufficient for floret development. In *Arabidopsis*, *LFY* directly activates *AP1* (Mandel and Yanofsky, 1995). *AP1* also promotes *LFY* transcription as part of a positive feedback loop (Kaufmann *et al.*, 2010). In addition, the FT–FD complex plays a role in up-regulating *FUL* and *AP1* expression in floral primordia (Wigge *et al.*, 2005). Since *FTL3* expression in the leaves was down-regulated by alteration in the photoperiod conditions [i.e. transfer from SD to NB conditions (Supplementary Fig. S5 at *JXB* online)], the down-regulation may be associated with *CsAFL1* and *CsM111* expression in the shoot apexes of *C. seticuspe* (Fig. 8). The weak *CsM111* expression related to the down-regulation of *CsFTL3* expression might modify *CsFL* up-regulation during floret development. This suggests that a similar signal network of the genes for flowering is conserved in chrysanthemums, as in *Arabidopsis*, and that there exists a possibility that in chrysanthemums, the photoperiodicity for capitulum primordia initiation and further development is tuned by *CsFTL3* in the leaves. This is supported by the fact that the *35S::CsFTL3* transgenic lines could overcome the inhibition of floral organ development under non-inductive LD conditions (Fig. 5).

In conclusion, the present study provides strong evidence that *CsFTL3* promotes floral transition in a similar manner to *FT-like* genes in other plant species. *CsFTL3* induction occurred in the leaves and its up-regulation was correlated with the events occurring in the SAM (i.e. floral evocation) that commit it to producing flowers (Evans, 1969) with the activation of floral integrator and/or floral identity genes. Further, *CsFTL3*-dependent graft-transmissible signals partially substituted for SD stimuli in chrysanthemum. Taken together, these observations indicate that the product of the *CsFTL3* gene could function as a florigen to regulate flowering, which has been previously demonstrated in studies on flowering physiology in chrysanthemum (Chailakhyan and Krikorian, 1975). It seems likely that the photoperiodic control of flowering may depend on the balance of promoters and inhibitors from leaves rather than on the accumulation of a threshold amount of a single floral stimulus at the shoot apex (Thomas and Vince-Prue, 1997). The observations suggest an argument that existence of inhibitors cannot be excluded and they might play an important role in the photoperiodic control of flowering. Further investigations to determine the role of *CsFTL* genes in the growth and flowering of chrysanthemums will provide clues for better understanding of the suggested argument.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Comparison of deduced amino acid sequences of CsFTL1, CsFTL2, CsFTL3, FT, and TFL1.

Figure S2. Expression of *CmFTL* genes in the leaves of Chrysanthemum morifolium 'Reagan' under SD and LD conditions.

Figure S3. Relative expression levels of exogenous *CsFTL3* mRNA in *in vitro* transgenic (*35S::CsFTL3*) Jimba plants.

Figure S4. Effect of *CsFTL3* overexpression on flowering in *Arabidopsis* under SD conditions.

Figure S5. Effect of transfer from SD to NB conditions on *CsFTL3* expression in *C. seticuspe*.

Table S1. Primer sequences and PCR conditions used in the study.

Table S2. Effect of defoliation on the transition from the vegetative to the reproductive phase in the plants resulting from grafting between wild-type (WT) and *35S::CsFTL3* transgenic plants.

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