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Molecular cloning and expression analyses of *FaFT*, *FaTFL*, and *FaAP1* genes in cultivated strawberry: their correlation to flower bud formation

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Abstract

In this study, we cloned flowering-related genes *FLOWERING LOCUS T* (*FT*) and *TERMINAL FLOWER1* (*TFL1*) from domesticated octaploid strawberries (*Fragaria* × *ananassa)* and analyzed their expression patterns in cultivars Tochiotome and Akihime. The floral meristem generation was induced under the short day and low temperature (SDLT), but not under the long day and high temperature (LDHT). We found that *FaFT1,* which is an orthologue of the *Arabidopsis* floral activator *FT*, was highly expressed in leaves under LDHT but not expressed in leaves under SDLT. On the other hand, the expression of *FaTFL2*, which belongs to the *TFL1* family of flowering repressing genes, decreased in crowns (stem tissue including meristem) under SDLT. These results suggest that *FaTFL2*, as opposed to *FvTFL1* in wild diploid strawberry *Fragaria vesca*, is related to flowering of the cultivated strawberry. Moreover, the *FaTFL2* expression might be regulated by temperature rather than by photoperiod. We demonstrated that a reduction of the *FaTFL2* expression is a key signal for flowering in domesticated strawberries.

Additional key words: flowering, *Fragaria* × *ananassa, F. vesca,* photoperiod, temperature.

Introduction

Most commercially important cultivars of strawberry (*Fragaria* × *ananassa*) are octaploids. There are two types of cultivated garden strawberries: one is an annual short day and low temperature-type (SDLT), and the other is the perpetual (everbearing) day-neutral or long day and high temperature-type (LDHT) (Sønsteby and Nes 1998, Taylor 2002, Sønsteby and Heide 2007, Weebadde *et al*. 2008, Bradford *et al*. 2010). To produce SDLT type strawberries from September to November, a super forcing cultivation system has been developed in Japan. In this system, plants are kept at the short day and cool temperature from the middle of July, and growers have to check the floral bud concealed in the crown (stem tissue including meristem) with a microscope to confirm floral bud formation after 30 to 35 d. However, it is quite difficult especially for inexperienced researchers or horticulturists to find floral buds of developing strawberries and also to judge their developmental stage. If a plant is transplanted and fertilized with nitrogen before floral bud formation, it often fails to differentiate floral buds which leads to late flowering and lowered yield.

For the photoperiodic control of flowering, *CONSTANCS* (*CO*) gene controls flowering through the activation of *FLOWERING LOCUS* T (*FT*) in LD plant *Arabidopsis thaliana* and its homologue *Heading date 3* in SD plant *Oryza sativa*. *FT* and *Heading date 3*, which are thought to be universal flowering signals, are activated when *CO* and its homologue *Heading date 1* expressions peak in late afternoon under LD conditions and after dusk under SD conditions (Hayama *et al*. 2003, An *et al*. 2004, Valverde *et al*. 2004, Corbesier *et al*. 2007). An accumulated FT or Heading date 3 protein in the phloem of leaves moves to the shoot apex where an FT/14-3-3/FLOWERING LOCUS D (FD) complex induces flowering by up-regulating floral meristem identity gene *APETALA1* (*AP1*) (Abe *et al*. 2005, Wigge

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Abbreviations: AP1 - APETALA1; CO - CONSTANCS; DTT - dithiothreitol; EGTA - ethyleneglycoltetraacetic acid; FD - FLOWERING LOCUS D; FT - FLOWERING LOCUS T; LDHT - long day and high temperature; LDLT - long day and low temperature; MSI1 - multicopy suppressor of IRA1; PEBP - phosphatidylethanolamine-binding protein; PVP - polyvinylpyrrolidone; RT- qPCR - real time quantitative polymerase chain reaction; SDHT - short day and high temperature; SDLT - short day and low temperature; SDS - sodium dodecyl sulfate; TFL1 - TERMINAL FLOWER1;
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et al. 2005, Corbesier *et al*. 2007, Jaeger and Wigge 2007, Tamaki *et al*. 2007, Taoka *et al*. 2011, Wigge 2011).

Floral repressor TERMINAL FLOWER 1 (TFL1) also binds to FD and suppresses the expression of *AP1* (Ratcliffe *et al*. 1999, Hanano and Goto 2011). *FT* and *TFL1* are homologous to animal phosphatidyl ethanolamine-binding proteins (PEBPs) including a Raf-kinase inhibitor protein (Yeung *et al*. 1999). In *Arabidopsis*, the *TFL1* expression is weak in the lower part of the apical meristem, but the protein can move short distances to repress flowering in the main apex (Conti and Bradley 2007).

Recently, it was shown that a 2-bp deletion in the wild strawberry *Fragaria vesca* homologue of *TFL1* (*FvTFL1*) is associated with perpetual flowering which demonstrates that *FvTFL1* is a key component of the perennial photoperiodic pathway in *F. vesca* (Iwata *et al*. 2012, Koskela *et al*. 2012). *FvTFL1*, not *FvFT1*, confers a short day requirement for flowering in *F. vesca* (Koskela *et al*. 2012). However, the genome construction of octaploid cultivated strawberry *F.* × *ananassa* is more complicated than that of wild strawberry *F. vesca*, and it is unknown whether the flower bud differentiation of cultivated strawberry is regulated by *TFL1*.

In this paper, we have succeeded in isolating *FT* and *TFLs* genes in cultivated strawberry *F.* × *ananassa* cv. Tochiotome, then we investigated their expression patterns to clarify the mechanism for floral bud formation in cultivated strawberries.

Materials and methods

Plants and cultivation: Runner plants of *Fragaria* × *ananassa* cvs. Tochiotome and Akihime having three expanded leaves were cut off and transferred to pots. Leaves were removed from the plants with the exception of three to five sets of the youngest fully expanded leaves. Plants were grown under short day and low temperature conditions (SDLT; an 8-h photoperiod and day/night temperatures of 31.2/15 °C) or long day and high temperature conditions (LDHT; a 14-h photoperiod and 29.8/27.2 °C). SDLT plants were grown in the field during daytime but they were placed into a curtained tunnel tent with air cooling (15 °C) from 17:00 to 9:00. Control plants were grown in the field. We sampled leaves and crowns of Tochiotome plants on 0, 5, 20, 30, and 35th day and of Akihime plants on 0, 5, and 35th day after the SDLT or LDHT treatments. The youngest expanded leaf was sampled from each plant at 17:00 at which *FaFT1* showed a clear LDHT-specific expression peak. Tissues were stored at -80 °C. For circadian rhythm experiments, samples of Tochiotome were placed in a growth chamber under LDHT (a 16-h photoperiod, 25/25 °C) or SDLT (an 8-h photoperiod, 25/15 °C) for 5 d. The youngest expanded leaf and crown were sampled every 4 h. For analyzing a temperature effect on strawberry flowering, we placed Tochiotome in a growth chamber under flower inductive LDLT (a 16-h photoperiod, 15/15 °C) and under expected flower non-inductive SDHT (an 8-h photoperiod, 25/25 °C) to find the correlation between *FaTFL2* expressions and flower bud formation. We sampled the crowns of plants on 0, 5, and 40th day after the LDLT and SDHT treatments. The developmental stages of the flower buds were defined as: stage 0 - an undifferentiated stage, stage A1-A3 - an initial to late differentiation stage, stage B1-B3 - an initial to late flower cluster formation stage, stage C - an initial sepal formation stage, stage D - an involucre formation stage.

RNA extraction: Total RNA was isolated by the hot borate method, as described in Wan and Wilkins (1994). Samples of about 0.1 g of frozen leaves or crowns were homogenized in liquid N_2 with an auto mill (*Tokken*, Chiba, Japan) and RNA was extracted with 1 cm^3 of hot (85 °C) borate buffer [(0.2 M sodium tetraborate decahydrate, 30 mM EGTA, 1 % (m/v) SDS, 1 % (m/v) sodium deoxycholate, 10 mM DTT, 1 % (v/v) *Triton X 114*, 2 % (m/v) PVP] and 2.5 mm^3 of proteinase K (1.375 unit; *Wako* pure chemical industry, Osaka, Japan).

Table 1. Primer sets for gene isolation, RT-PCR, and quantitative real-time PCR (qPCR) analyses. Primers APIF, APIR, MSI1F, and MSI1R are from Mouhu *et al*. 2009.

Primer	Oligonucleotides (5'→3')
Gene isolation	
5FaFT1	ATCACCCAATTAGTTGTTAG
3FaFT1	ATTAGTAAAGGGTTTACGATGATC
5FaTFL1-1	CCCTCTTTCGAGTTCTAACA
FaTFL1-R	TTTGGGATCTGGCCTGCCTC
5FaTFL2	GCTTCCTCAAGGAAAACAGA
3FaTFL2	CACCAAGAGCATCGATCACT
5FaTFL3	TGATACTGCTGCACTTCTTC
3FaTFL3	TCATCTTCTTCTGGCTGCAG
RT-PCR, qPCR	
FaFTF common	TGAGCTCAAACCTTCCCAAG
FaFT1R	ATTAGTAAAGGGTTTACGATGATC
FaFT2R	GAGAATAATTACTAAGACAATTCTACA
FaFT3R	CATAATACACAGGATTACATTATAG
FaTFL1F	CACCTCGACTGGATTGTGAC
FaTFL1R	TTTGGGATCTGGCCTGCCTC
FaTFL2F	TGACAGTGACTTACAACTCC
FaTFL2R	CCTCCCTTCCAAATGTGTTG
FaTFL3F	ACGAGATCATGCCTTCTGTC
FaTFL3R	ACTTCTCTTCCAAAGGAGAC
AP1F	CGCTCCAGAAGAAGGATAAGG
AP1R	CATGTGACTGAGCCTGTGCT
actinF	TGCTCCCGCTATGTATGTTG
actinR	GGACTTCTGGGCATCTGAAA
MSI1F	TCTCCACACCTTTGATTGCCA
MSI1R	ACACCATCAGTCTCCTGCCAAG

Genomic DNA was removed by using a RNase free DNase I kit according to the manufacturer's instruction (*TaKaRa*, Shiga, Japan).

Isolation of *FaFT1*, *FaTFL1-1*, *FaTFL2*, and *FaTFL3*: The cDNA was reverse-transcribed from RNA extracted from leaf (*FaFT1*) or crown (*FaTFLs*) tissues of strawberry cv. Tochiotome using a *PrimeScript* RT reagent kit (with *Perfect Real Time* gDNA eraser) (*TaKaRa*) with primers designed on the basis of the *F. vesca* reference genome (Shulaev *et al*. 2011). The primers used are described in Table 1. The reaction was conducted in a 25 mm^3 mixture containing 1 mm^3 of cDNA (10 ng), 200 µM each deoxynucleotide, 1 µM each primer, and 1.25 units of *Ex Taq* DNA polymerase (*TaKaRa*). Reaction conditions were 35 cycles at 98 °C for 10 s, 58 °C for 30 s, and 72 °C for 1 min in a thermal cycler (*GeneAmp 2720*, *Applied Biosystems*, Tokyo, Japan). Each amplified fragment was cloned into the *T-vector pMD20* (*TaKaRa*) by ligation and sequence was determined by performing dideoxy chain termination sequencing on an *Applied Biosystems 3130* genetic analyzer using a *BigDye Terminator v. 3.1* cycle sequencing kit (*Life Technologies*, Tokyo, Japan).

Expression analyses (RT-PCR): cDNA was synthesized from 300 ng of total RNA using a *PrimeScript* RT reagent kit with gDNA eraser (*TaKaRa*). RT-PCR was performed using *Go Taq Master Mix* and polymerase (*Promega*, Osaka, Japan). The primer sets for the expression analysis are listed in Table 1. *Actin* and *MSI1* were used as housekeeping genes to calculate the relative expression. PCR conditions were initial heating at 95 °C for 2 min followed by 33 cycles for *FaFT1*, *FaFT2*, and *FaFT3*, 23 cycles for actin, and 25 cycles for *MSI1* consisting of 95 °C for 30 s, 58 °C for 30 s , and 72 °C for 30 s. Gel images were quantified by the *Image J* software (Abramoff *et al*. 2004).

Quantitative real-time PCR (qPCR) for the quantification of transcripts of *FaTFL1-1* and *FaTFL2* was conducted using *SYBR Premix Ex Taq* (*Tli RNaseH Plus*; *TaKaRa*) and the thermal cycler *Dice TP800* real time system (*TaKaRa*). *MSI1* was used as housekeeping gene (Mouhu *et al*. 2009). cDNA was synthesized using a *PrimeScript* RT reagent kit with gDNA eraser (*TaKaRa*). The primer sets are listed in Table 1. PCR conditions were preheating at 95 °C for 30 s followed by 45 cycles of 95 °C for 5 s, 60 °C for 10 s, and 72 °C for 25 s. The relative expression was calculated using the thermal cycler real time system software *Dice v. 3.00D* (*TaKaRa*). Three independent RNA preparations per stage were used for analyses, and data are shown as mean ± SE of three samples for each stage.

Results and discussion

We found eight genes belonging to the *FT*/*TFL1* family (mrna04680.1, mrna09405.1, mrna13304.1, mrna13481.1, mrna21535.1, mrna21992.1, mrna28959.1, and mrna30276.1) in the *Fragaria vesca* reference genome by searching with the protein name of PEBP (Shulaev *et al*. 2011). According to deduced amino acids sequences and a phylogenetic tree, all genes except for mrna09405.1 and mrna13481.1 belong to either the *FT* (mrna04680.1, mrna21535.1 and mrna28959.1) or *TFL1* (mrna13304.1, mrna21992.1, and mrna30276.1) family. The corresponding *FT*/*TFL1* genomic fragments from the cultivated strawberry *Fragaria* × *ananassa* we named *FaFT1* (corresponding to mrna21535.1), *FaFT2* (corresponding to mrna04680.1), *FaFT3* (corresponding to mrna28959.1.1), *FaTFL1-1* (corresponding to mrna30276.1), *FaTFL2* (corresponding to mrna21992.1), and *FaTFL3* (corresponding to mrna13304.1) according to their similarity with those in *F. vesca*.

We isolated *FaFT1*, *FaTFL1-1*, *FaTFL2*, and *FaTFL3* cDNA clones from the leaf or crown of *F.* × *ananassa* cv. Tochiotome, but we could not obtain *FaFT2* and *FaFT3* cDNA from either leaves or crowns. In the case of *FaTFL3*, six amino acids from the C-terminal end are derived from primer (3FaTFL3) sequence. The *FaFT1* cDNA clone contained a 528-bp coding region encoding 176 amino acids with M_r and pI values of 19.741 and 8.42, respectively. These are closely related to *Arabidopsis FT* with 175 amino acids with M_r and pI values of 19.808 and 7.96, respectively. The *FaTFL1-1*, *FaTFL2*, and *FaTFL3* cDNA clones contained 516-bp, 519-bp, and 525-bp coding regions encoding 172, 173, and 175 amino acids with M_r and pI values of 19.311 and 9.19, 19.463 and 9.88, and 19.750 and 8.73, respectively. These are also closely related to *Arabidopsis TFL1* with 177 amino acids with M_r and pI values of 20.157 and 10.10, respectively. The sequences of *FaFT1*, *FaTFL1-1*, *FaTFL2*, and *FaTFL3* from Tochiotome have been submitted to DDBJ under accession numbers AB840273, AB822995, AB822996, and AB840275, respectively.

The number of deduced amino acids of *FaFT1* from cv. Tochiotome (176) differed from that of *F. vesca FvFT* (199; Fig. 1). *FaFT1* did not have 22 N-terminal amino acids existing in *FvFT* since the putative initiation codon ATG was preceded by a stop codon TGA at a 10 - 12 bp upstream region in the frame. The homology between *FaFT1* and *FvFT* cDNAs was 68.2 % at the deduced amino acid level. *FaFT1* showed the same amino acid sequence with *FvFT* CDS (mrna 21535.1). The *FaTFLs*, *FaTFL1-1*, *FaTFL2*, and *FaTFL3* exhibited 61.7 - 79.2 % similarities, and *FaTFL1-1* showed a high similarity with *FvTFL1-1*/*1-2*/*1-3* (99.4 - 100 %) suggesting that *FaTFL1-1* is a *FvTFL* homologue. *FvTFL1-1*, *1-2*, and *1-3* seemed to be alleles in *F. vesca* since the deduced amino acids sequences of *FvTFL1-1* and *1-2* were the same, and that in *FvTFL1-3* there was only one amino acid difference from *FvTFL1-1* and *1-2*. In agreement with the critical

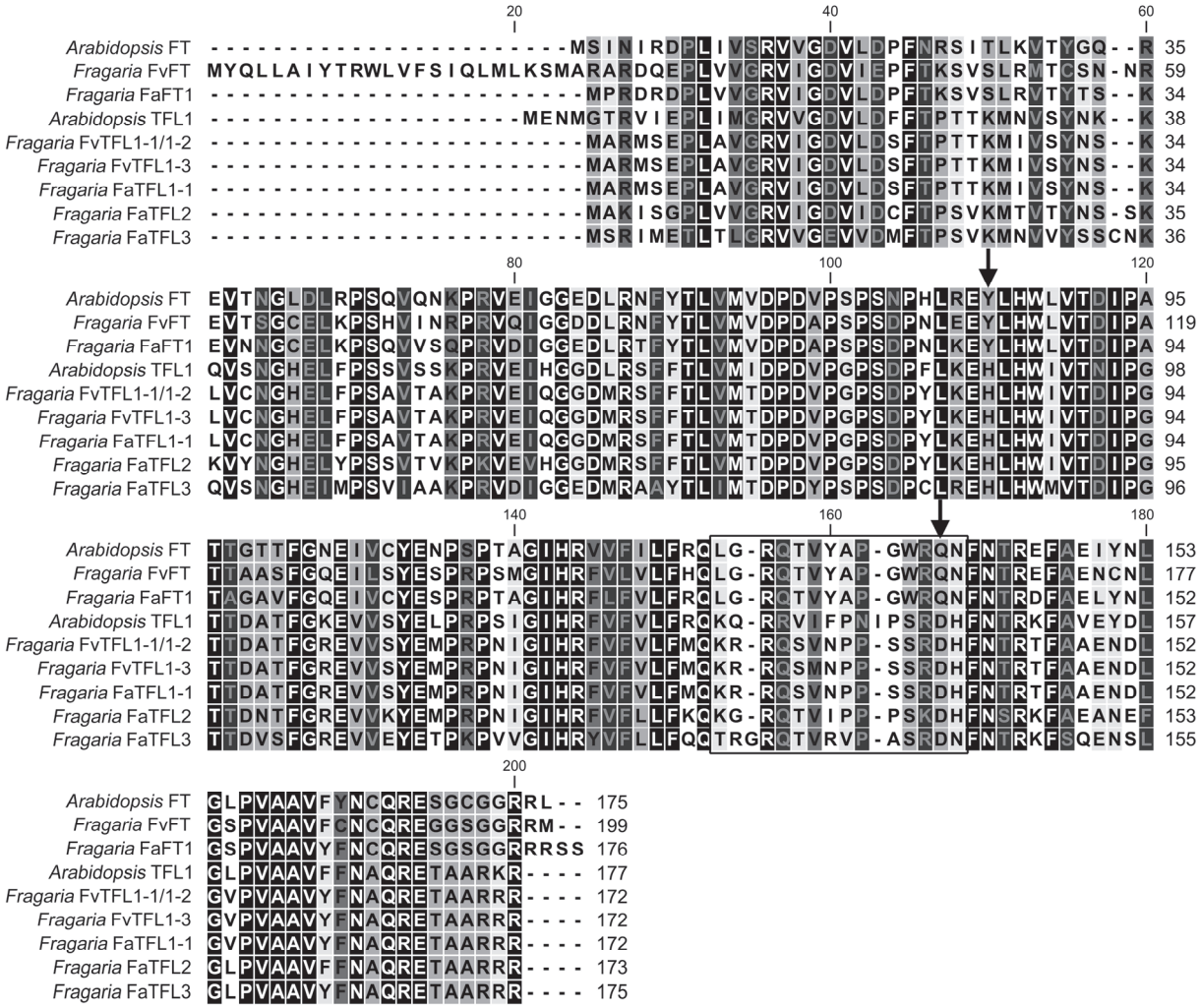

Fig. 1. The alignment of deduced amino acids sequences of FT from *Arabidopsis thaliana* cv. Columbia (AF152096.1), FvFT from *Fragaria vesca* (CBY25183), FaFT1 from *Fragaria* × *ananassa* cv. Tochiotome (AB840273), TFL1 from *A. thaliana* (AAB41624), FvTFL1-1 and 1-2 (KSN) from *F. vesca* (JN172097 for 1-1 and HQ378595 for 1-2), FvTFL1-3 (SOC1) from *F. vesca* (JF806631), FaTFL1-1 from *F.* × *ananassa* (AB822995), FaTFL2 from *F.* × *ananassa* (AB822996), and FaTFL3 from *F.* × *ananassa* (AB840275). The gaps introduced to facilitate the alignment are presented with the *dashes*. Amino acids in *black* and in *white* are 100 and 50 % or less of similarity, respectively, in the nine members of the FT/TFL family. The similarity of the amino acids of *gray* gradation is for 50 to 100 %. The *arrows* indicate amino acid positions related to antagonistic functions between *FT* and *TFL1* (Hanzawa *et al*. 2005, Ahn *et al*. 2006). The *box*, called segment B, represents the region of a potential ligand-binding pocket in FT/TFL1 family proteins (Ahn *et al*. 2006).

amino acid difference between *Arabidopsi*s FT and TFL1 (Hanzawa *et al*. 2005, Ahn *et al*. 2006), FaFT1 had Tyr 84 and Gln 139 but FaTFL1-1, FaTFL2, and FaTFL3 had His 84 and Asp 139, His 85 and Asp 140, and His 87 and Asp 142, respectively (Fig. 1).

To confirm the relationships among the isolated cDNA clones, we constructed a phylogenetic tree with *FvFT*, *FvTFLs*, *Malus MdFT1*, *MdTFL1* (Mimida *et al*. 2012), and *Arabidopsis AtFT* and *AtTFL1* (Fig. 2). The phylogenetic tree was divided into two major clades. As expected, *FaFT1* grouped together with *FvFT*, *MdFT1*, and *AtFT* in an *FT* clade. *FaTFL1-1*, *FaTFL2*, and *FaTFL3* grouped together with *FvTFL1-1/1-2/1-3, MdTFL1*, and *AtTFL1* in an *TFL* clade. From the results, *FaFT1* and *FaTFLs* (*FaTFL1-1, FaTFL2*, and *FaTFL3*) seem to be members of *FT* and *TFL*, respectively.

FT gene, also called florigen, acts as floral activator (Turck *et al*. 2008, Turnbull 2011). Spatial gene expression analyses of *FvFT1* and *FvFT2* show the highest expression in leaves and flower buds (Koskela *et al*. 2012). *FvFT1* expression in leaves of both SD *F. vesca* (PI551792; National Clonal Germplasm Repository) and LD *F. vesca* cv. Hawaii-4 was observed in seedlings under LD, but not SD conditions, and *FvFT1* show a clear LD-specific expression peak during the night (Koskela *et al*. 2012). As the *FvFT1* expression is down-regulated in SD *F. vesca* (PI551792), it acts as floral activator only in cv. Hawaii-4 (Koskela *et al*. 2012).

Table 2. The time-course expressions of *FaFT*, *FaTFL*, and *FaAP1* genes in leaves and crowns of *F.* × *ananassa* cvs. Tochiotome and Akihime grown under different photoperiods and temperatures for 0 to 35 d. The transcription relative to housekeeping genes *Actin* for *FaFTs*, and *MSI1* for *FaTFLs* and *FaAP1*. Mean levels ± SE, $n = 3$.

Gene	Cultivar	Tissue	Treatment	0 d	5 d	20 d	30 d	35 d
FaFT1	Tochiotome	leaves	LDHT	0.43±0.34	0.80±0.4	1.18±0.35	0.74±0.22	0.85±0.24
	Tochiotome	leaves	SDLT	0.43±0.34	0.08±0.02	0.10±0.05	0.09±0.01	0.09±0.02
FaFT2	Tochiotome	leaves	LDHT	0.05±0.02	0.05±0.02	0.06±0.03	0.07±0.02	0.06±0.02
	Tochiotome	leaves	SDLT	0.05±0.02	0.06±0.01	0.07±0.02	0.08±0.01	0.08±0.02
FaFT3	Tochiotome	leaves	LDHT	0.06±0.02	0.06±0.02	0.09±0.02	0.10±0.02	0.08±0.01
	Tochiotome	leaves	SDLT	0.06±0.02	0.07±0.01	0.12±0.03	0.11±0.02	0.08±0.01
FaTFL1-1	Tochiotome	crowns	LDHT	1.00±0	2.17±0.63	1.50±0.47	1.80±0.50	1.86±0.75
	Tochiotome	crowns	SDLT	1.00±0	1.98±0.56	1.13±0.39	1.33±0.43	1.83±0.61
FaTFL2	Tochiotome	crowns	LDHT	1.00±0	2.20±0.48	2.18±0.64	2.50±1.03	1.17±0.28
	Tochiotome	crowns	SDLT	1.00±0	3.58±0.34	1.06±0.09	0.49±0.03	0.40±0.13
FaTFL3	Tochiotome	crowns	LDHT	0.74±0.22	1.00±0.06	0.85±0.09	1.16±0.06	0.55±0.07
	Tochiotome	crowns	SDLT	0.74±0.22	1.04±0.10	0.65±0.07	0.79±0.07	0.62±0.05
FaAP1	Tochiotome	crowns	LDHT	0.63±0.24	0.21±0.08	0.63±0.21	0.38±0.12	0.22±0.04
	Tochiotome	crowns	SDLT	0.63±0.24	0.24±0.02	0.28±0.18	0.12±0.05	1.18±0.15
FaTFL1-1	Akihime	crowns	LDHT	1.00±0	0.87±0.10	-	-	1.28±0.15
	Akihime	crowns	SDLT	1.00±0	0.88±0.19	-	-	1.61±0.35
FaTFL2	Akihime	crowns	LDHT	1.00±0	1.00±0.16	-	-	2.02±0.53
	Akihime	crowns	SDLT	1.00±0	1.11±0.25	-	-	0.25±0.01

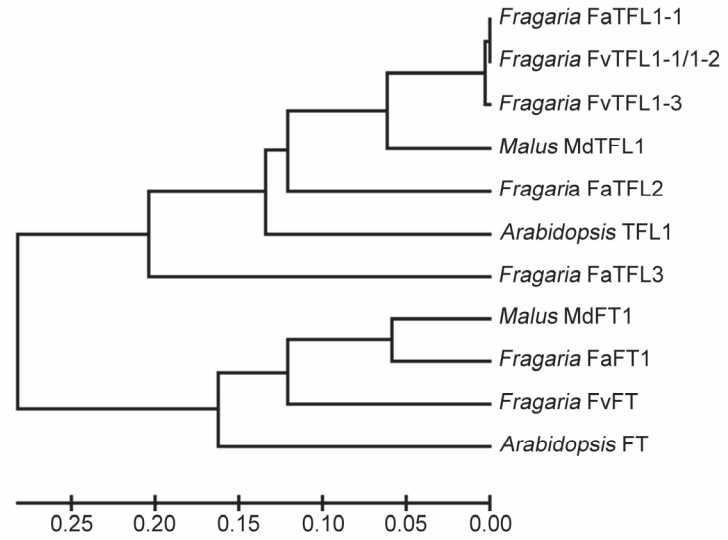

Fig. 2. A dendrogram generated using the unweighed pair group method with an arithmetic average (UPGMA) analysis showing relationships between FT/TFL family proteins as shown in Fig. 1 with MdFT1 (AB161112) and MdTFL1 (AB052994) from *Malus* × *domestica*.

Table 3. The circadian rhythm of *FaFT1* expression determined by RT-PCR in leaves of *F.* × *ananassa* cv. Tochiotome grown under LHDT or SDHT conditions for five days. Means ± SE, $n = 3$. *Actin* was used as stable control gene.

Treatments	00:00	04:00	08:00	12:00	16:00	20:00	24:00
LDHT	0.32±0.11	2.41±1.16	3.52±1.85	1.59±1.12	3.32±0.87	1.23±0.86	0.26±0.02
SDLT	0.11±0.02	0.39±0.1	0.45±0.15	0.41±0.18	0.30±0.06	0.19±0.05	0.15±0.05

We investigated the spatial and temporal expressions of *FaFTs* in cv. Tochiotome by RT-PCR analyses. From time-course expression analyses, *FaFT1* was expressed continuously under the LDHT, but not under SDLT conditions (Table 2). The *FaFT1* expression was diminished 5 d after the SD-treatment (Table 2). In the cases of *FaFT2* and *FaFT3*, we could not detect an expression in leaves under both LDHT and SDLT (Table 2). The circadian rhythm of the *FaFT1* expression was observed under LDHT, but not under SDLT similarly as in *F. vesca* (Table 3). *FaFT1* showed clear LDHT-specific expression peaks during a day and at the beginning of dark (Table 3). The time course of *FaFT1* expression is different from that of *FvFT1* with only one peak at night (Koskela *et al*. 2012). This difference may reflect different genetic backgrounds between cultivars. Moreover, no *FaFT1*, *FaFT2*, and *FaFT3* expressions were observed in crown tissues under the LDHT and SDLT conditions including 30 and 35 d of the SDLT treatment at which flower buds were observed (results not shown). Thus, *FaFT1* seems not to act as florigen unlike in other SD plants (Hayama *et al*. 2003, 2007, Kong *et al*. 2010).

FvTFL1 is expressed highly in the crown under LD, whereas it is expressed only weakly in the crown three weeks after a SD treatment (Koskela *et al*. 2012). The *FvTFL1* reduction is slightly earlier than the flower bud formation occurring after four weeks (Koskela *et al*. 2012). In SD-grown *F. vesca*, *FvTFL1* is strongly down-regulated before the activation of the floral meristem identity genes *APETALA1* (*AP1*) and *FRUTIFULL* (*FUL*) and before flower initiation. The reduction of *FvTFL1* mRNA content in SD *F. vesca* by RNAi silencing induces flowering under non-inductive LD conditions (Koskela *et al*. 2012). From the results, the photoperiodic control of *FvTFL1* mRNA levels in the shoot apex is a primary mechanism to control the photoperiodic induction of flowering in SD *F. vesca* (Koskela *et al*. 2012).

We investigated the *FaTFL1-1* expression, which is an orthologue of *FvTFL1,* in the crown of cv. Tochiotome under flower inductive SDLT and flower non-inductive LDHT by qPCR analyses. No reduction of *FaTFL1-1* expression was observed with the passage of time under both LDHT and SDLT (Table 2). This suggests that *FaTFL1-1* is not a candidate for a flowering repressor in the cultivated strawberry. *FaTFL3* also showed a similar time-course to *FaTFL1-1*. Contrarily, the *FaTFL2* expression was reduced after 30 and 35 d at SDLT (Table 2) compared to day 0. Interestingly, the expression of *FaTFL2* was up regulated 3.6 times at 5 d after the SDLT treatment and then reduced below one-seventh at 30 to 35 d. We observed flower buds of stages 0 to A3 on some samples after 30 d (in some samples, it was difficult to recognize undifferentiated or just beginning floral bud formation) and on all samples after 35 d (stages A2 to C) and also a clear up regulation of *FaAP1* expression at 35 d after the SDLT treatment (Table 2). Therefore, *FaTFL2*, but not *FaTFL1-1* and *FaTFL3*, seems to be a candidate for a floral repressor in cv. Tochiotome. In the case of cv. Akihime, we confirmed flower bud differentiation of stages A3 to B3 at 35 d after the flower inductive SDLT treatment. As the reduction of *FaTFL2*, not *FaTFL1-1*, at 35 d after the SDLT treatment was observed not only in Tochiotome but also in Akihime (Table 2), this *FaTFL2* reduction might be characteristic for the annual SDLT types of cultivated strawberries.

Table 4. *FaTFL1-1* and *FaTFL2* expressions analyzed by qPCR in crowns of *F.* × *ananassa* cv. Tochiotome grown under different treatments for 0, 5, and 40 d. Flower buds were observed at 35 or 40 d after transfer to LDLT and SDHT, respectively. The transcription relative to housekeeping gene *MSI1*. Means ± SE, $n = 3$.

Gene	Treatments	0 d	5 d	40 d
FaTFL1-1	LDLT	1.00±0	1.33±0.36	0.98±0.11
	SDHT	1.00±0	0.86±0.27	0.65±0.29
FaTFL2	LDLT	1.00±0	0.12±0.05	0.08±0.03
	SDHT	1.00±0	0.24±0.10	0.16±0.03

In SDLT grown strawberries, temperature might be a more important factor for flowering than day length. Indeed, floral bud differentiation occurs under LD conditions after July with cooling the crown below 20 °C (K. Dan, personal communication). We grew cv. Tochiotome under the LDLT and SDHT conditions and confirmed the floral buds differentiation of stage B1 at 40 d after the LDLT treatment as expected, however, we also observed floral buds of stages A3 to B2 at 35 d after the SDHT treatment (unpublished results). Increasing temperature to 24 °C considerably reduces flowering annual SD cultivars (Verheul *et al*. 2006 and our unpublished results), but keeping the constant temperature at 25 °C did not repress floral buds induction in Tochiotome. The *FaTFL1-1* expression was slightly reduced at 40 d after the LDLT and SDHT treatments, but the *FaTFL2* expression was markedly reduced already at 5 d after the LDLT and SDHT treatments, and this low expression was until floral buds formation time (40 d). As the reduction of the *FaTFL2* expression under the LD conditions was observed at low temperature, its reduced expression seems to be controlled by temperature. Under the SD conditions, the *FaTFL2* expression was markedly or slightly increased at 5 d after the SDLT treatment in both the cultivars (Table 2), but markedly reduced at 5 d after SDHT in Tochiotome (Table 4). These differences might be due to different temperatures 15 and 25 °C in LDLT and SDHT, respectively. The delay of the *FaTFL2* repression seemed to be caused by a temperature higher than 30 °C in SDHT during the day (Verheul *et al*. 2006). A reduced *FaTFL2* expression under the SD conditions seemed to be controlled by temperature.

In conclusion, the reduction of the *FaTFL2* expression in cultivated strawberries correlated with flower induction caused by SDLT, LDLT, or SDHT. Although the photoperiodic repression of *FvTFL1* expression correlated

with the floral initiation of wild strawberry, the repression of *FaTFL2*, not *FaTFL1-1* (homologue of *FvTFL1*) and *FaTFL3*, seems to correlate with floral initiation in cultivated strawberry. The *FaTFL2* expression of cultivated strawberry seems to be regulated by temperature rather than photoperiod.

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