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Environmental responses of the *FT/TFL1* gene family and their involvement in flower induction in *Fragaria* × *ananassa*

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ABSTRACT

Flowering time control is important for fruit production in *Fragaria* × *ananassa*. The flowering inhibition pathway has been extensively elucidated in the woodland strawberry, *Fragaria vesca*, whereas the factors involved in its promotion remain unclear. In this study, we investigated the environmental responses of *F.* × *ananassa FT* and *TFL1*-like genes, which are considered key floral promoters and repressors in many plants, respectively. A putative floral promoter, *FaFT3*, was up-regulated in the shoot tip under short-day and/or low growth temperature, in accordance with the result that these treatments promoted flowering. *FaFT3* mRNA accumulated before induction of a floral meristem identity gene, *FaAP1*. *FaFT2*, a counterpart of *FvFT2*, expressed in the flower bud of *F. vesca*, was not induced in the shoot tip differentiating sepal or stamen, suggesting that this gene works at a later stage than stamen formation. In *F. vesca*, *FvFT1* transmits the long-day signal perceived in the leaves to the shoot tip, and induces the potent floral inhibitor *FvTFL1*. *FaFT1* was expressed in the leaves under long-day conditions in *F.* × *ananassa*. Expression of *FaTFL1* was higher in the shoot tip under long-day than short-day conditions. Independent of day-length, *FaTFL1* expression was higher under high temperature than low temperature conditions. These results suggest that *FaFT3* induction by short-day or low temperature stimuli is a key step for flowering initiation. As in *F. vesca*, *F.* × *ananassa* floral inhibition pathways depend on *FaTFL1* regulation by day-length via *FaFT1*, and by temperature.

Introduction

The cultivated strawberry, *Fragaria* × *ananassa* ($2n = 8x = 56$), is an important perennial crop. *F.* × *ananassa* cultivars can be roughly divided into two groups, seasonal flowering (SF) and everbearing (EB), according to their flowering habits (reviewed by Heide et al., 2013). SF cultivars are classified into facultative or obligate SD plants (Sønsteby and Heide, 2006). Flowering of SF cultivars is promoted by SD conditions during autumn. Generally, floral transition is inhibited under warm conditions (e.g. above 25 °C) or occurs under cool conditions (e.g. below 15 °C) independently of photoperiod (Ito and Saito, 1962; Heide et al., 2013). Anthesis occurs in the following spring under natural conditions, because reproductive growth is promoted by LD and warm temperature (Heide et al., 2013). On the other hand, in EB cultivars, flowering occurs perpetually without SD and cold stimuli, and is promoted under LD (Heide et al., 2013). In Japan, application of artificial flowering initiation (e.g. cooling and SD treatments) and subsequent promotion (e.g. heating and LD treatments) to SF cultivars are performed for early and large fruit production. EB cultivars have been used to produce fruits, particularly during summer, the changeover period for SF cultivars.

In many plants, the time of floral initiation is governed by floral promoters and repressors that respond to environmental and endogenous cues. A floral promoter that is produced under floral-inductive photoperiods in the leaves, and functions in the shoot apical meristem (SAM), is named a florigen (Chailakhyan, 1936). Recent molecular genetic studies have demonstrated that genes encoding phosphatidylethanolamine-binding protein (PEBP), such as FLOWERING LOCUS T (FT) in *Arabidopsis thaliana*, and its orthologues in several plant species, act as the systemic floral inducers, also called 'florigens' (Lifschitz et al., 2006; Corbesier et al., 2007; Lin et al., 2007; Tamaki et al., 2007). Subsequent to the proposal of

Abbreviations: ATC, *Arabidopsis thaliana* CENTRORADIALIS homologue; BFT, BROTHER OF FT AND TFL1; CEN, CENTRORADIALIS; EB, everbearing; FL, fluorescent lamp; FT, FLOWERING LOCUS T; IL, incandescent lamp; LD, long day; PEBP, phosphatidylethanolamine binding protein; SAM, shoot apical meristem; SD, short day; SF, seasonal flowering; SOC1, SUPPRESSOR of the OVEREXPRESSION of CONSTANTS 1; TFL1, TERMINAL FLOWER 1; zt, zeitgeber time.

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the florigen concept, a floral inhibitor 'antiflorigen', which is produced in the leaves under non-floral-inductive photoperiods, was also proposed (Lang and Melchers, 1943). Recently, a systemic floral repressor that matches the concept proposed by Lang and Melchers was found in *Chrysanthemum seticuspe*. *C. seticuspe Anti-florigenic FT/TFL1 family protein*, (*CsAFT*) is transcribed and translated mainly in the leaves under non-floral-inductive photoperiods, and inhibits flowering (Higuchi et al., 2013). *FT* and *CsAFT* encode a small globular protein with similarity to PEBP. There are six members of the PEBP gene family in *Arabidopsis*. Among the FT/TFL1-like proteins, FT and TWIN SISTER OF FT act as floral activators (Kardailsky et al., 1999; Kobayashi et al., 1999; Yamaguchi et al., 2005), whereas TERMINAL FLOWER 1 (TFL1), BROTHER OF FT AND TFL1 (BFT) and the *A. thaliana* CENTRORADIALIS (CEN) homologue (ATC) have been shown as floral inhibitors (Bradley et al., 1997; Mimida et al., 2001; Yoo et al., 2010; Huang et al., 2012). *TFL1* and *BFT* are mainly expressed in the SAM to inhibit flowering and to maintain indeterminate growth of the inflorescence, but are unlikely to be involved in photoperiodic flowering responses (Ratcliffe et al., 1998; Yoo et al., 2010; Hanano and Goto, 2011). *ATC* acts as a systemic floral inhibitor under SD that is mainly expressed in vascular tissues of petioles, hypocotyls, and roots (Huang et al., 2012). Because further understanding of day-length and temperature responses of flowering is important for stable fruit production in SF *F.* × *ananassa*, molecular genetic studies are in progress to reveal the regulation of flowering.

F. × *ananassa* seems to be produced by hybridization between *Fragaria chiloensis* and *Fragaria virginiana*. Recent studies have shown that *Fragaria vesca*, a diploid woodland strawberry ($2n = 14$), is one of the most probable ancestor of *F.* × *ananassa* (Rousseau-Gueutin et al., 2009; Isobe et al., 2013). Genetic studies in *F. vesca* have shown that perpetual flowering is caused by recessive alleles of a single repressor gene called *SEASONAL FLOWERING LOCUS* (*SFL*, Brown and Wareing, 1965). Runnering is a trait that negatively affects flowering and caused by the dominant *RUNNERING* locus in *F. vesca* (*RU*, Brown and Wareing, 1965). Although both *F.* × *ananassa* and *F. vesca* show similar environmental responses, they appear to differ in the genetic control of the perpetual flowering trait. It has been shown that perpetual flowering is controlled by a single dominant locus (Morishita et al., 2012) in *F.* × *ananassa*. One major quantitative trait locus, named *FaPFRU*, has been identified in *F.* × *ananassa*, which is not orthologous to *SFL* and *RU* in *F. vesca* (Gaston et al., 2013). However, *FaPFRU* positively and negatively links to perpetual flowering and runnering, respectively (Gaston et al., 2013). Taken together, there seems to be at least two mechanisms for perpetual flowering in *Fragaria*: floral inhibition by *Fa/FvTFL1* and a floral promotion mechanism, acting independently of *Fa/FvTFL1*.

Recently, it has been reported that *SFL* encodes a homologue of *TFL1* in *F. vesca* (Iwata et al., 2012; Koskela et al., 2012; reviewed by Kurokura et al., 2013). Mutation in a *TFL1* homologue of the SF *F. vesca* (*FvTFL1* or *FvKSN*) results in perpetual flowering, suggesting that this gene is the key floral repressor governing the SF habit. *FvTFL1* is highly expressed in the shoot tip under LD and down-regulated under SD (Koskela et al., 2012). *FvTFL1* is also down-regulated by cool temperatures even under LD (Kurokura et al., 2013), suggesting its involvement in the day-length-independent floral initiation by cool temperatures. In *F. vesca*, the *FT*-like gene (*FvFT1*) is strongly expressed in leaves under LD, in both SF and EB cultivars (Koskela et al., 2012; Rantanen et al., 2014). The floral promoter function of *FvFT1* has been confirmed in EB cultivars (Koskela et al., 2012; Rantanen et al., 2014). In *F.* × *ananassa*, Thompson and Guttridge (1960) showed that a defoliated SF cultivar can initiate flowering independent of photoperiod, suggesting the presence of an LD-induced floral inhibitor in leaves. Although *FvFT1* promotes flowering in an EB accession of *F. vesca* lacking functional *FvTFL1* (Koskela et al., 2012; Rantanen et al., 2014), Mouhu et al. (2013) have reported that *FvFT1* can play an LD-induced mobile floral inhibitor role in SF accessions. In *Arabidopsis, SUPPRESSOR of the OVEREXPRESSION of CO1* (*SOC1*) is one of the downstream targets of *FT* and promotes flowering (Michaels et al., 2005). However, in *F. vesca*, *FvSOC1* seems to act as a floral inhibitor by inducing *FvTFL1* (Mouhu et al., 2013).

Thus, the mechanisms involved in the floral inhibition pathways determining the SF trait have been elucidated to a large degree in *F. vesca*. However, it is still unclear whether there is a key floral promoter that is induced under floral-inductive conditions in SF accessions. Here, the effects of temperature and photoperiod on the expression of the *FT/TFL1*-like gene were studied to determine the SF mechanism of *F.* × *ananassa* by using the SF cultivar 'Nyoho'.

Materials and methods

Plant materials

An SF cultivated strawberry, *Fragaria* × *ananassa* cv. 'Nyoho' (Akagi et al., 1985), was used for the experiment. The stolons were rooted in 7.5-cm plastic pots filled with Metro-Mix 350 (Sun Gro Horticulture, Agawam, MA), supplemented with delayed-release fertilizer (IBS1, National Federation of Agricultural Cooperative Association, Tokyo, Japan) at the beginning of August. In addition to natural light, 4 h of light exposure (from 23:00 (h) to 03:00 (h)) with incandescent lamps (IL, K-RD100V75W/D, Panasonic Corporation, Osaka, Japan) was provided as a night break to inhibit flowering. At the end of August, the plants were acclimated in a growth chamber (LPH-350SP, NK system, Osaka, Japan) at 27 °C and 70% relative humidity for 3 d under LD. The photoperiod consisted of an 8-h main light period supplied via fluorescent lamps (FL, FHF32EX-N-HX-S; NEC Lighting Ltd. Tokyo, Japan) at a photosynthetic photon flux density (PPFD) of 200 $\mu mol\,m^{-2}\,s^{-1}$. For the LD treatment, 8-h day-length extensions were supplied via incandescent lamps at a PPFD of 40 $\mu mol\,m^{-2}\,s^{-1}$, because red plus far-red light is efficient for flowering inhibition in *F.* × *ananassa* (Vince-Prue and Guttridge, 1973). SD treatment was performed with an 8-h main light period. Temperature and day-length treatments were performed in a chamber at 13, 20, or 27 °C under LD or SD conditions for 35 d. After the treatments, leaves were dissected and shoot apices were observed under a stereomicroscope.

Sequence analysis

Amino acid sequences of *Arabidopsis* (BROTHER OF FT AND TFL1 (BFT), NM125597; *A. thaliana* CENTRORADIALIS (CEN) homologue (ATC), AB024714; FT, AF152096; MFT, AF147721; TSF, AF152907; TFL1, U77674) were used to search the genome sequences of *F. vesca* (Shulaev et al., 2011), using the tblastn algorithm. The PCR primers are designed based on *CEN/FT/TFL1*-like sequences of *F. vesca* obtained by a local BLAST (Supplementary Table S1). Direct PCR cloning was performed using ExTaq (TaKaRa BIO Inc., Shiga, Japan) and a cDNA template synthesized from the mRNA of various organs of *F.* × *ananassa*. After being subcloned into a pGEM Teasy vector (Promega KK, Madison, WI), the DNA was sequenced using a BigDye Terminator Cycle Sequencing Kit v. 3.1 and an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA). Amino acid sequences were aligned using ClustalX (Thompson et al., 1997) with the PAM protein weight matrix (Supplementary Fig. S1). Neighbour-joining analysis was performed using MEGA5 software (Tamura et al., 2011) with the Jones–Taylor–Thornton matrix.

Total RNA preparation and reverse-transcription

Fully expanded leaves and shoot tips (ca. 5 mm in length) were frozen in liquid nitrogen. The leaves were collected after 7 d and the shoot tips were collected after 7, 21, and 35 d of photoperiod and temperature treatments. The samples were ground into a fine powder using a Shake Master homogenizer (Bio Medical Science Inc., Tokyo, Japan). Total RNA was extracted from the frozen powder using the cetyltrimethylammonium bromide method (Yu et al., 2012), treated with RNase-Free DNase (Qiagen K.K., Tokyo, Japan), and purified by using an RNeasy Mini Kit (Qiagen K.K.). The purity and concentration of total RNA were measured using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific K. K., Kanagawa, Japan). Reverse transcription (RT) was performed using 500 ng of total RNA and PrimeScript RT Master Mix Perfect Real Time (TaKaRa BIO Inc.), according to the manufacturer's instructions. The resulting cDNA was diluted to 10% with TE buffer.

Quantitative PCR

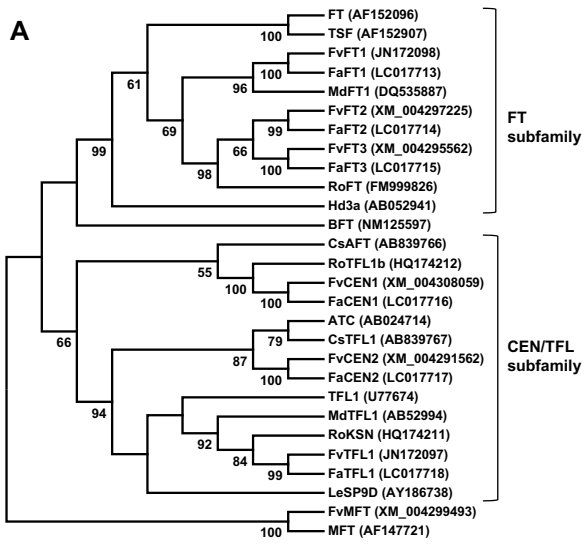
cDNA was prepared from three independent biological replicates and analysed by two PCR technical replicates. Quantitative PCR was performed by using SYBR Premix Ex Taq II Tli RNaseH plus (TaKaRa BIO Inc.) in a Thermal Cycler Dice Real Time System II (TaKaRa BIO Inc.). Cycling conditions were as follows: 1 min of denaturation at 95 °C, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. The *ACTIN* gene (*FaACT*, LC017712) was amplified as an internal standard to normalize the raw data. The PCR primers used in this study are listed in Supplementary Table S1. It was confirmed that the cycle threshold of *FaACT* did not differ considerably among the samples (means ± standard deviation were 23.4 ± 0.3 in whole-leaf samples and 23.1 ± 0.5 in whole-shoot tip samples).

Results

Identification of FT/TFL1 homologue in F. × ananassa cv. 'Nyoho'

Six CEN/FT/TFL1-like putative amino acid sequences were picked from the *F. vesca* genome sequences, which were used to isolate *F.* × *ananassa* counterparts. Two sequences, which had already been annotated as *FvFT1* (JN172098) and *FvFT2* (XM_004297225), and another sequence (XM_004295562), fell into the clade that includes FT and TSF (Fig. 1A). cDNA was cloned from *F.* × *ananassa* and referred to as *FaFT1* (LC017713), *FaFT2* (LC017714), and *FaFT3* (LC017715), respectively. FaFT2 and FaFT3 are most closely related to RoFT, a putative floral promoter in the rose (Randoux et al., 2013). An *F.* × *ananassa* counterpart of FvTFL1 (JN172097) was also cloned as FaTFL1 (LC017718). A phylogenetic analysis revealed that the putative amino acid sequence of XM_004308059 (annotated as CENTRORADIALIS-like protein 1, FvCEN1), together with RoTFL1b (HQ174212) of rose, belongs to a large clade of CEN/TFL1 (Fig. 1A). FvCEN1 is closely related to CsAFT (AB839766), and separated from the CEN and TFL1 clades, which include FvTFL1 and RoKSN (HQ174211), a TFL1 counterpart in rose. Here, a counterpart of *FvCEN1* in *F.* × *ananassa* was named *FaCEN1* (LC017716). A counterpart of XM_004291562, which has also been annotated as CEN-like (*FvCEN2*), was cloned from *F.* × *ananassa* and was referred to as *FaCEN2* (LC017717).

There are several amino acid residues in FT/TFL1-like proteins, whose necessity for the floral promoter function have been elucidated by comparisons between *Arabidopsis* FT and TFL1 (Hanzawa et al., 2005; Ahn et al., 2006). A Tyr residue in the FT subfamily (Tyr85 in FT) and a His in the TFL1 subfamily (His88 in TFL1) are highly conserved and indispensable for floral promotion and repression, respectively (Hanzawa et al., 2005). The corresponding residues in Fa/FvFT proteins were Tyr, while those in Fa/FvTFL1 and FaCEN-like proteins were His (Fig. 1B). There are regions in the vicinity of the C terminus, called segment B in FT/TFL1 family proteins (Ahn et al., 2006). Gln residues in segment B of the FT subfamily (Q140 in FT) are also important for floral promotion (Ahn et al., 2006). The corresponding residues in Fa/FvFT proteins are Gln (Fig. 1B). On the other hand, those in Fa/FvCEN-like and Fa/FvTFL1 proteins are Asp. Ho and Weigel (2014) identified the amino acid residues indispensable for floral promoter function in the external loop of FT. Residues corresponding to Y134, W138, and N152 in FT are conserved in Fa/FvFTs, but not in Fa/FvTFL1 and Fa/FvCENs.



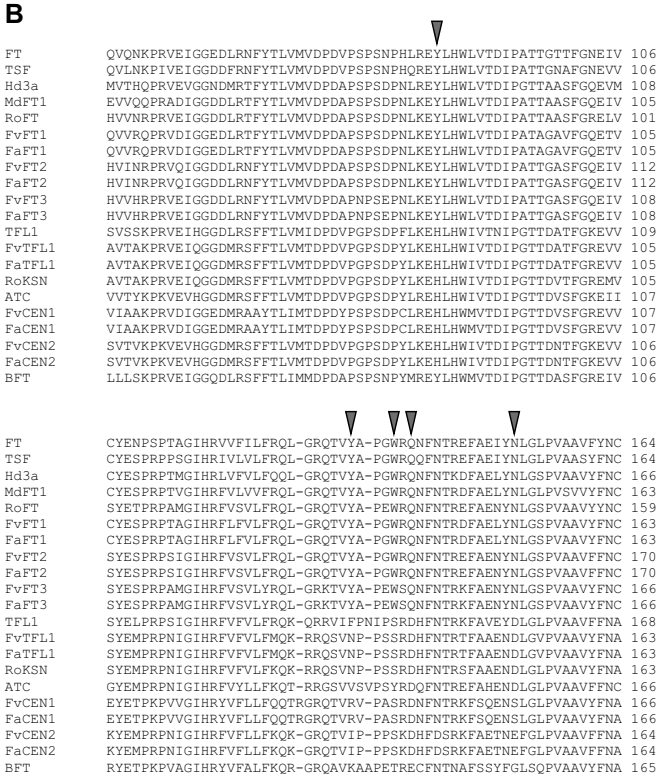


Fig. 1. (A) Phylogenetic analysis of several PEBP family proteins. Bootstrap values exceeding 50% derived from 1000 resamplings are indicated below the branches. (B) Partial sequence alignment of PEBP family proteins. Amino acid residues essential for floral promoter function are indicated by arrows.

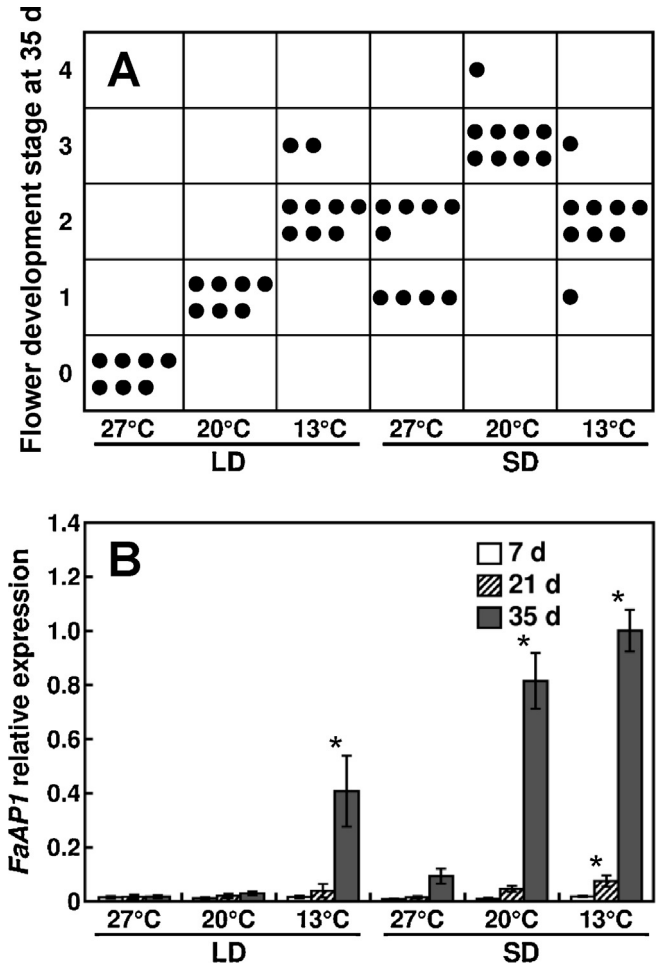


Fig. 2. Effects of temperature and photoperiod on flowering (A) and *FaAP1* expression (B). (A) After 35 d of environmental treatment, the shoot tips were classified into five stages (Taylor et al., 1997): 0: vegetative growth, 1: doming, 2: floral meristem differentiation, 3: sepal differentiation, 4: stamen differentiation. Seven to nine shoot tips were observed. One dot represents one dissected plant. (B) Gene expression analyses were performed using the *FaACT* gene as an internal standard. The shoot tip samples were collected after 7, 21, and 35 d of treatment at zeitgeber time (zt) 2–3. The highest value was set at 1.0. Bars are means ± SE ($n = 3$). * Significantly larger than 27 °C/LD at the same day in the shoot tip by Dunnett's test ($P \leq 0.05$).

Effects of photoperiod and temperature on flowering and AP1-like gene expression in F. × ananassa cv. 'Nyoho'

After 35 d of temperature and photoperiod treatments, flowering stages were observed under a microscope (Fig. 2A). After 35 SDs, plants initiated visible floral meristem differentiation at 27 °C, which was not observed under LD. Faster flower development was observed at 20 °C/SD than at 20 °C/LD and 27 °C/SD. These are in accordance with the promotion of floral initiation by SD and cool temperature (Ito and Saito, 1962; Taylor, 2002). The plants continued vegetative growth when cultivated at 27 °C/LD. Swelling of the SAM was observed in the plants grown at 27 °C/SD and at 20 °C/LD. The plants differentiated immature stamen at 20 °C/SD. The plants were at the sepal-forming stage when cultivated at 13 °C for 35 d both under SD and LD, in accordance with the photoperiod independent floral promotion by cool temperature (Ito and Saito, 1962).

After 35 d of treatment, expression of *FaAP1* (JN788263), a counterpart of *FvAP1*, probably involved in the maintenance of floral meristem identity (Koskela et al., 2012), was significantly higher in the plants grown at 20 °C/SD, 13 °C/LD, and 13 °C/SD (Fig. 2B) than at 27 °C/LD. In terms of floral developmental stages, significant induction of *FaAP1* was observed in SAMs differentiating the inflorescence meristem, but not in those at the doming stage (Fig. 2A and B). Thus, *FaAP1* expression seems to be an indicator of flower development after inflorescence meristem formation.

Effects of photoperiod and temperature on FT/TFL1-like gene expression in F. × ananassa cv. 'Nyoho'

FaFT1 expression in the leaves showed a daily oscillation that peaked at zt 0 or16 under LD (Fig. 3A). The level was significantly lower under SD than under LD at all of the time points tested. It was expressed at much lower levels in the shoot tip than in the leaves (Fig. 3F). None of the treatments induced significant *FaFT1* expression in the shoot tip. Expression of *FaFT2* in the leaves peaked at zt 0 at 27 °C/LD and 20 °C/LD, and none of the environmental treatment induced significant changes in *FaFT2* expression (Fig. 3B), seen in the effect of day-length on *FaFT1* expression. *FaFT2* expression in the shoot tip became slightly higher at 35 d under 20 °C/LD, 13 °C/LD, and 13 °C/SDs than under 27 °C/LD (Fig. 3G). *FaFT3* expression in the leaves became higher at zt 0 and 8 than at zt 16, and was unaffected by environmental conditions (Fig. 3C). *FaFT3* expression in the shoot tip at 21 d became significantly higher in the plants grown at 20 °C/SD or 13 °C/SD (Fig. 3H). It was also highly expressed at 35 d in the plants grown at 13 °C/LD. A slight induction of *FaFT3* was also observed in the plants grown at 27 °C/SD. Expression of *FaFT3* in the shoot tip significantly increased before *FaAP1* induction in the plants grown at 20 °C/SD and 13 °C/SD (Figs. 2B and 3H). In the plants grown at 27 °C/SD and 13 °C/LD, induction of both *FaFT3* and *FaAP1* was observed simultaneously at 35 d.

Compared to 7 d, *FaTFL1* expression significantly decreased at 21 d in all the conditions examined (Fig. 3I). At 35 d, the expression became lower at 20 °C/SD, 13 °C/LD, and 13 °C/SD than at 27 °C/LD. *FaTFL1* expression in the leaves tended to be higher at zt 0 and to decrease during the light period (Fig. 3D). However, it was much lower than that in the shoot tips and did not respond to environmental treatments (Fig. 3D). Expression of *FaCEN1* was nearly at the same levels in the leaves (Fig. 3E). At 35 d, *FaCEN1* expression became lower at 20 °C/LD, 13 °C/LD, 27 °C/SD, and 20 °C/SD than at 27 °C/LD (Fig. 3J). Similarly, *FaCEN2* expression did not differ among the environmental treatments in the shoot tip (Supplementary Fig. S2), and was hardly detected in the leaves.

Discussion

The function of FT/TFL1-like proteins in *F. × ananassa* was examined, based on their amino acid sequences. FaFT1, FaFT2, and FaFT3 fell into the clade that includes FT protein (Fig. 1A). The amino acid residues required for the floral promoter activity of *Arabidopsis* FT were conserved in these polypeptides (Fig. 1B). Moreover, this clade includes MdFT1 of *Malus × domestica* and FvFT1 of *F. vesca* (Fig. 1A). Overexpression of *MdFT1* in *M. × domestica* and *FvFT1* in *F. vesca* induced early flowering (Tränkner et al., 2010; Koskela et al., 2012), indicating the floral promoter function of these genes in Rosaceae plants. These suggest that FaFTs could act as floral promoters in *F. × ananassa*. FaTFL1 belongs to the same clade as TFL1 and is separated from the clade that includes ATC, FaCEN1, and FaCEN2 (Fig. 1A). Corresponding amino acids of FaTFL1, FaCEN1, and FaCEN2 were not identical to those in FT, some of which were identical to those in TFL1 (Fig. 1B). This suggests that FaTFL1 and FaCENs are possible floral inhibitors.

It has been reported that *FvTFL1* is a key inhibitor of flowering under LD and warm temperatures in SF *F. vesca* (Koskela et al., 2012; Iwata et al., 2012). *FvTFL1* is highly expressed in the shoot tip during vegetative growth and decreases under SD in SF *F. vesca* (Koskela et al., 2012). *FaTFL1* expression levels at the end

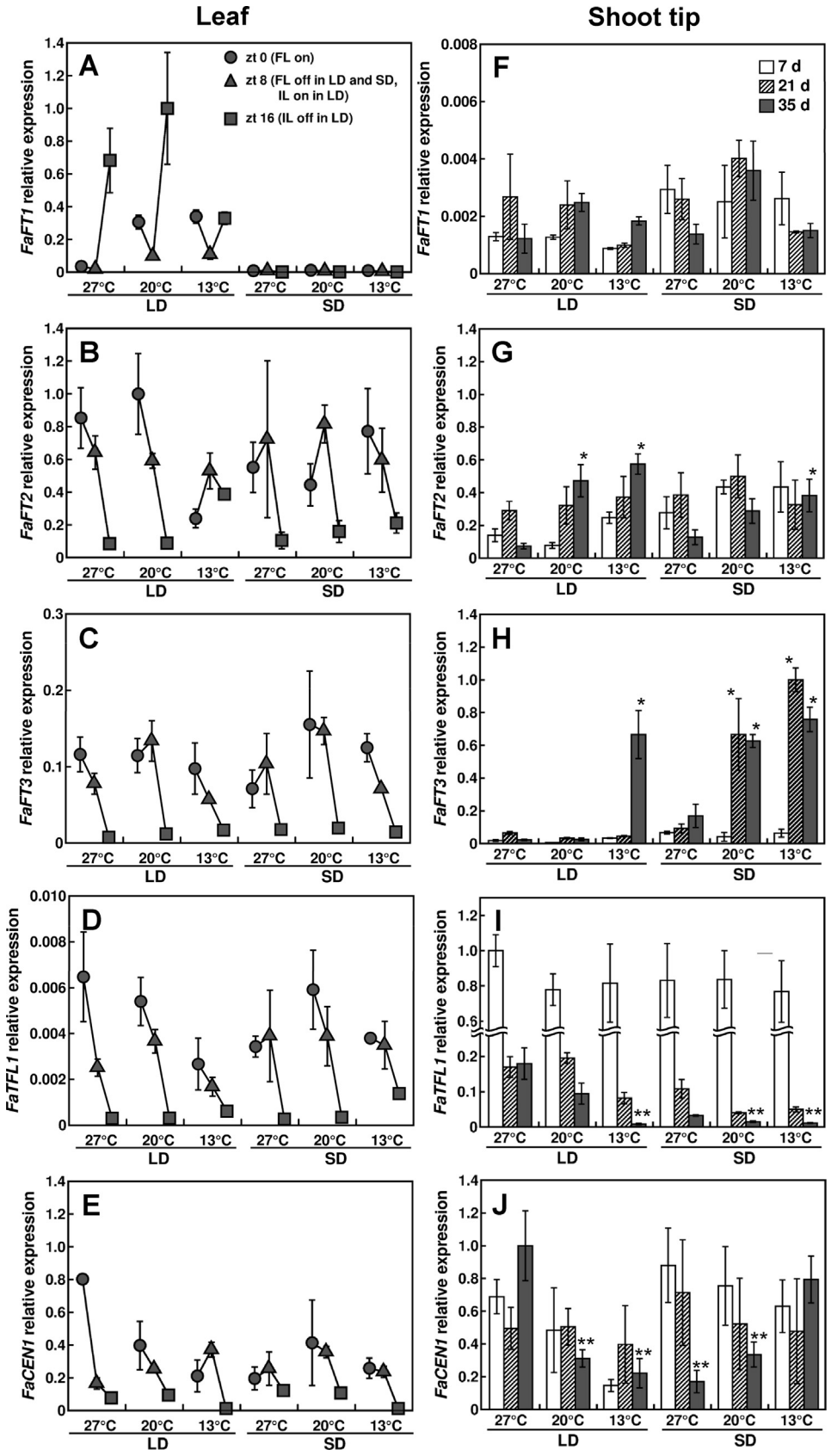


Fig. 3. Effects of temperature and photoperiod on *FT/TFL1-like* genes in the leaves (A–E) and shoot tips (F–J). Gene expression analyses were performed using the *FaACT* gene as an internal standard. The highest value in each target was set at 1.0. Bars are means ± SE ($n = 3$). (A–E) The leaf samples were collected after 7 d of treatment at zt 0, 8, and 16. (F–J) The shoot tip samples were collected after 7, 21, and 35 d of treatment at zt 2–3. *, ** Significantly larger (*) and smaller (**) than 27 °C/LD at the same day in the shoot tip by Dunnett's test ($P \leq 0.05$).

of the experiment became lower at 27 °C and 20 °C under SD than at these temperatures under LD (Fig. 3I). Kurokura et al. (2013) have shown that *FvTFL1* was down-regulated by cool temperatures even under LD. In terms of temperature responses under LD, *FaTFL1* expression at 35 d was significantly lower at 13 °C than at 20 °C and 27 °C (Fig. 3I). *F.* × *ananassa* typically grows vegetatively under LD and moderate temperatures. In some SF *F.* × *ananassa* cultivars, high temperature inhibits floral initiation independent of day-length (Ito and Saito, 1962). In this study, only 27 °C/LD inhibited floral initiation completely (Fig. 2A), indicating that the other environments in the growth chamber were insufficient for obligate floral inhibition. In accordance with this, after transferring the plants from the glasshouse to a growth chamber, *FaTFL1* tended to be down-regulated after 21 days in all conditions investigated (Fig. 3I). Temperatures below 27 °C or artificial LD seem less inductive for *FaTFL1* than the natural high temperatures and high light intensities of the Japanese summer, when the experimental plants were prepared. However, an essential quantitative correlation between *FaTFL1* expression level and flowering suggest that this gene is a key floral repressor in *F.* × *ananassa*, as well as in *F. vesca*: negative correlation was observed between flowering (flower development and *FaAP1* expression) and *FaTFL1* expression (Figs. 2 and 3I).

Recently, *CsAFT* was identified as a systemic floral inhibitor produced in the leaves under LD or NB in an SD plant, chrysanthemum (Higuchi et al., 2013). Among the *FT/TFL1*-like sequences in *F.* × *ananassa*, *FaCEN1* encodes a possible floral inhibitor most closely related to *CsAFT* (Fig. 1A). However, *FaCEN1* did not show leaf-specific expression or photoperiod responses (Fig. 3E and J), indicating that it is not a *CsAFT*-like systemic floral inhibitor. Although it showed opposite temperature responses in the shoot tip under SD and LD at the last time point, no correlation was observed between *FaCEN1* expression and flower development (Figs. 2A and 3J).

The EB habits of *F. vesca* caused by the *FvTFL1* null allele show recessive inheritance (Iwata et al., 2012). However, EB habits are controlled by a single dominant gene in some *F.* × *ananassa* (Morishita et al., 2012; Gaston et al., 2013), suggesting that there are key floral promotion mechanisms dominant to *FaTFL1* in *F.* × *ananassa*. *Fa/FvFT1* was mainly expressed in the leaves of SF *F.* × *ananassa* (Fig. 3A), and SF and EB *F. vesca* (Koskela et al., 2012; Mouhu et al., 2013) under LD, which is a non-floral inductive condition for SF cultivars. The floral promoter function of *FvFT1* has been shown in EB cultivars lacking *FvTFL1* (Koskela et al., 2012; Mouhu et al., 2013; Rantanen et al., 2014). The role of *FvFT1* has yet to be determined in SF cultivars, because LD conditions induce *FvTFL1* expression. It has been suggested that in SF cultivars, *FvFT1* is transported to the SAM and up-regulates *FvTFL1* by way of *FvSOC1* induction (Mouhu et al., 2013). Although an *SOC1* homologue that is widely known as one of the downstream targets of FT and floral promoter in *Arabidopsis* (Samach et al., 2000), *FvSOC1* is considered a floral inhibitor in SF *F. vesca* (Mouhu et al., 2013). In this study, *FaFT1* expression levels were higher under LD than under SD, regardless of temperature conditions (Fig. 3A). The day-length response of *FaSOC1* expression was not appreciable at 7 and 21 d, whereas it tended to become higher under LD than SD at 35 d (Supplementary Fig. S3). *FaTFL1* expression levels at the end of the experiment remained higher under LD than under SD at 27 °C and 20 °C (Fig. 3I). This suggests that although *FaTFL1* regulation through a photoperiod pathway involving *FaFT1* seems to be conserved in *F.* × *ananassa*, the involvement of *FaSOC1* in the pathway could not be clarified by the expression analysis in this study. In terms of temperature responses, expression of *FaFT1* and *FaSOC1* did not differ among the temperatures under LD (Fig. 3A, Supplementary Fig. S3), but *FaTFL1* expression decreased at 13 °C (Fig. 3I). Thus, the cool temperature pathway for *FaTFL1* down-regulation seems dominant to the photoperiod pathway involving *FaFT1* and *FaSOC1*. TFL1-like proteins inhibit flowering by antagonizing FT-like proteins when forming flowering promoter complexes with FD protein (Hanano and Goto, 2011; Higuchi et al., 2013; Randoux et al., 2013). The same mechanism has been proposed in SF *F. vesca* (Koskela et al., 2012). Association of FaFT1 with FD-like protein might be antagonized by FaTFL1. The floral promoter activity of FaFT1-FD might be masked in the presence of FaTFL1-FD in the shoot tip of SF *F.* × *ananassa*. Accordingly, the role of Fa/FvFT1 should be studied further.

Expression of *FvFT2* is several hundred-fold higher in flower buds than in leaves and crown (Koskela et al., 2012). In this study, such drastic induction of *FaFT2* was not observed within 35 d, even in the shoot tip of the plants differentiating stamens, which had already accumulated the *FaAP1* transcript (Figs. 2A and B and 3G). These results suggest that *FaFT2* works downstream of *FaAP1*, and at a later stage of flower development than stamen differentiation.

FaFT3 was induced in the shoot tip under SD or cool temperatures, and might act upstream of *FaAP1* (Figs. 2B and 3C and H). Conditions conducive to the induction of *FaFT3* are repressive for *FaTFL1* (Fig. 3H and I). FaFT3-FD might promote flowering in the absence of FaTFL1 under SD. In many plants, *FT-like* genes are predominantly induced in the leaves in response to a floral-inductive photoperiod within a few days. It takes time before induction of *FaFT3* occurs under floral-inductive conditions in the shoot tip, and no photoperiod responsive expression was observed in the leaves (Fig. 3C and H). This suggests that *FaFT3* is not a systemic floral promoter directly responding to SD stimuli, but rather it is induced as a result of photoperiod signaling from the leaves and temperature sensing in the SAM. *FaFT3* induction occurred even under LD at 13 °C, whereas the expression became lower at 27 °C than at other temperatures under SD (Fig. 3H). Thus, *FaFT3* regulation by temperature seems dominant to that by photoperiod. In the LD plant *Beta vulgaris*, which has two *FT* homologues in its genome, *BvFT1* probably inhibits flowering by repressing and antagonizing a floral promoter, *BvFT2*, under SD (Pin et al., 2010). In addition, it has also been suggested that *BvFT1* inhibits *BvFT2* induction before cold exposure (Pin et al., 2010). In *Pisum sativum*, shoot apex-specific expression of *FTa1* and *FTc* is essential for flowering time regulation. They are regulated by *FTa1* and *FTb*, photoperiod-responsive mobile signals from the leaves (Hecht et al., 2011). In *C. seticuspe*, a transient expression assay showed that expression of *CsFTL3* was up-regulated by *CsFTL3/CsFDL1* co-expression (Higuchi et al., 2013). These observations suggest that FT/TFL1-like proteins could regulate not only the expression of flowering-related genes, such as *APETALA1* and *FRUITFUL* (Abe et al., 2005; Wigge et al., 2005), but also that of the *FT*-like gene. There is a possibility that *FaTFL1* induced in the SAM might inhibit *FaFT3* expression under LD or warm temperatures.

Our results suggest that *FaFT3* and *FaTFL1* in the SAM of SF *F.* × *ananassa* seem to play an important role in flower induction controlled by environmental cues. Expression of these genes in the SAM seems to be regulated by photoperiod signalling from the leaves, and more dominantly by temperature. *FaFT3* induction by short-day or low temperature stimuli seems to be a key step for flowering initiation. As in *F. vesca*, *FaTFL1* regulation by LD via *FaFT1*, and by warm temperature, seems to be an important floral inhibition pathway in *F.* × *ananassa*. The timing and duration of flowering are major factors contributing to crop production. Further studies are required to understand the function and regulation of *FaFT3* and *FaTFL1*.

Nucleotide sequences have been deposited in the DDBJ/EMBL/GenBank databases under the accession numbers LC017712 (*FaACT*), LC017713 (*FaFT1*), LC017714 (*FaFT2*), LC017715 (*FaFT3*), LC017716 (*FaCEN1*), LC017717 (*FaCEN2*), and LC017718 (*FaTFL1*).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jplph.2015.01.007.

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